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UNIVERSITY OF ALBERTA

The Effect of Inflammatory Mediators and Salbutamol on Calcium Mobilization, Cyclic AMP and Isometric Tension in Bovine Tracheal Smooth Muscle.

BY

Andrew James Hirsh ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

IN

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EDMONTON, ALBERTA
Spring 1993

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January 29th, 1993

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *The Effect of Inflammatory Mediators and Salbutamol on Calcium Mobilization, Cyclic AMP and Isometric Tension in Bovine Tracheal Smooth Muscle* submitted by **Andrew James Hirsh** in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine.

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January 28th, 1993

To my wife Katherine, your love and motivation is an everlasting inspiration.

Abstract

Leukotriene D_4 (LTD_4), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), platelet-activating factor (PAF) and histamine are inflammatory mediators that have been implicated in the pathogenesis of asthma. Acute airway narrowing is one of the pathophysiological features of asthma and $ß_2$-agonist therapy is a pharmacological modality commonly used to reverse bronchospasm. The effect these inflammatory mediators have on airway smooth muscle contraction and the mechanism of ß-agonist relaxation in airway smooth muscle have not been clearly defined; however, the second messengers, adenosine 3', 5', cyclic monophosphate (cyclic AMP) and intracellular calcium concentrations ($[Ca^{++}]_i$), have been closely associated with isometric tension.

In the first chapter of this thesis, smooth muscle morphology, the role of second messengers ($[Ca^{++}]_i$ and cyclic AMP) in the contraction/ relaxation mechanism of airway smooth muscle, pharmacology of inflammatory mediators and salbutamol (a $ß_2$-agonist), and techniques for simultaneous measurement of $[Ca^{++}]_i$ and isometric tension are reviewed.

In the second chapter, LTD_4, $PGF_{2\alpha}$, PAF and histamine were examined for their ability to generate a reproducible isometric tension-time curve in bovine trachealis muscle. LTD_4, $PGF_{2\alpha}$, PAF and histamine all cause isometric contraction, but the tension-time curves for LTD_4, $PGF_{2\alpha}$, and PAF were not reproducible. $PGF_{2\alpha}$, showed a sustained tension but was not dose-dependant. LTD_4 and PAF both caused a transient increase in tension and were not reproducible. Histamine showed a dose-dependant relationship and the response was reproducible.

In the third chapter, the fluorescent indicator, fluo-3, was used to measure $[Ca^{++}]_i$ in bovine trachealis muscle during histamine-induced isometric contraction. Fluo-3 has no significant modifying effect of its own on the isometric contraction caused by histamine, and is a good indicator of $[Ca^{++}]_i$ changes during histamine-induced contraction. Finally, histamine caused a sustained increase in both $[Ca^{++}]_i$ and isometric tension.

In the fourth chapter, the effects of salbutamol on simultaneous measurements of either 1) cyclic AMP, 2) $[Ca^{++}]_i$ or 3) extracellular calcium concentration ($[Ca^{++}]_{ext}$), and isometric tension in bovine tracheal smooth muscle were shown. Salbutamol caused a decrease in $[Ca^{++}]_i$ and isometric tension and increased both cyclic AMP and $[Ca^{++}]_{ext}$ during a histamine-induced contraction.

Acknowledgement

This thesis would not have been possible without the help from Dr. Peter Pang, for the use of his CAF-100 Flourimeter; Dr. Don Enarson and Dr. Dale Lein for the use of their computers; Dr. Tom Ryan, Anh Nguyen and Bill Boyd for their technical assistance and the friendly group at Gainers Inc. (especially the men on the kill floor) (Edmonton, AB. CANADA) for suppling the bovine trachea.

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Chapter I

Introduction

Introduction

Statistical evidence has determined that there is a significant increase in the worldwide asthma mortality rate (Markowe *et al.*, 1987; Jackson *et al.*, 1988). This suggests that our current pharmacological therapy for asthma may be inadequate, and improved therapy necessitates a better understanding of the pathogenesis of this condition.

The three major manifestations of asthma are bronchospasm, inflammation, and increased mucus secretion. This literature review focusses on the first two manifestations by concentrating on the second-messengers {intracellular calcium and adenosine 3', 5', cyclic monophosphate (cyclic AMP)} and how their intracellular concentrations may control the airway smooth muscle contractile process.

Airway smooth muscle is found throughout the tracheobronchial tree. The percentage of the airway wall composed of smooth muscle increases from the large airways (trachea) to the small airways (terminal bronchioles) (Russell, 1978), but the mechanical characteristics between tracheal smooth muscle and bronchial smooth muscle (to the 5th generation bronchi) were found to be similar (Stephens *et al.*, 1969). Because the mechanical characteristics of both tracheal and bronchial smooth muscle are similar, and that tracheal smooth muscle is present in greater quantity and more easily isolated, trachealis smooth muscle was chosen as the tissue for my study.

1. *Airway Smooth Muscle Morphology*

The orientation of the trachealis muscle in relation to other tissue types, within the trachea is depicted in figure 1.1. The posterior aspect of the trachea has a thick band of smooth muscle (muscularis trachea) which is attached via elastic tendons to the perichondrium of the cartilaginous rings of the trachea. The smooth muscle is joined to the mucosa by loose connective tissue and inserted among dense elastin and collagenous fibre bundles. The blood supply comes from the inferior thyroid artery. The nerves acting on the muscle arise from the recurrent branch of the vagus and the sympathetic chain (Bloom and Fawcett, 1987). A delicate network of lymphatics is present in the thick basal lamina covered, in the luminal side, by ciliated pseudostratified columnar epithelium.

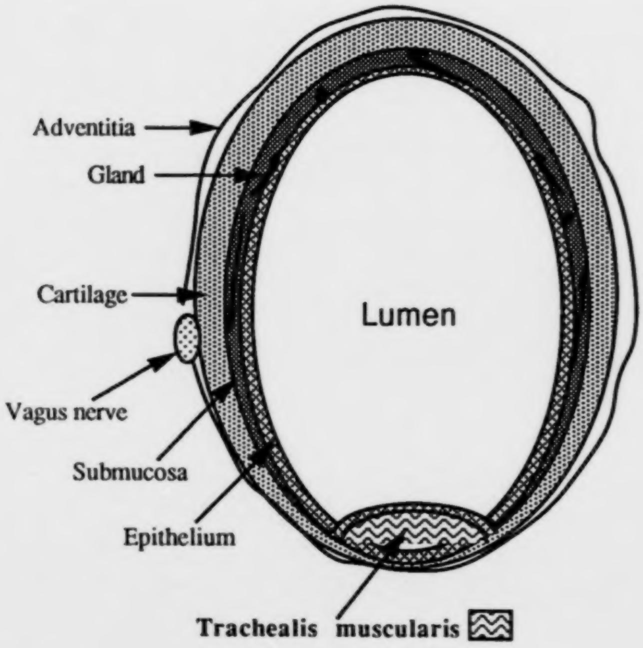

Figure #1.1 Trachea morphology (modified from Sorokin, 1983)

At the cellular level, airway smooth muscle cells are long and narrow, ranging from 750 -1,000 μM long x 5 μM thick (Stephens, 1987 A; Suzuki *et al.*, 1986) and are arranged parallel to each other. They have a limited nerve supply (1 nerve filament for every 10 muscle cells) (Stephens, 1987 A) and are separated by an interfascicular space containing collagen and elastin fibres. A smooth muscle bundle is composed of approximately 5% fibroblasts (Stephens, 1987 A) and within the bundle the cells have relatively few gap junctions. According to Burnstock, (1970) these features classify the airway smooth muscle as neither a multiunit nor a single unit type; instead airway smooth muscle can be classified as an intermediate type (Stephens, 1987 B).

Airway smooth muscle cells contain the two main contractile proteins, actin and myosin, which are similar to those of the skeletal muscle. However, airway smooth muscle contains only 1/5 the myosin content of skeletal muscle and the myosin filaments are 20% longer. Although there are no visible striations, tracheal smooth muscle does show some form of sarcomere organization (Stephens, 1987 B). To give the cell structural integrity, the contractile protein actin has been shown (histologically) to run through dense bodies found in the myoplasm and the actin filaments are also attached to dense bands which are part of the sarcolemma (Stephens, 1987 B).

2. *Role of Airway Smooth Muscle on Airway Calibre*

Because airway smooth muscle, like skeletal muscle, has the ability to contract against a force, and is found surrounding the tracheobronchial tree, a reduction in length of the muscle (reducing the radius of the lumen) has a significant deleterious effect on airflow to and from the gas-exchange units of the lungs. The resistance to flow through a non-collapsible tube equals: **[(8) x (length of the tube)(viscosity of air)] / [(π) x (radius^4)]** (Forster II *et al.*, 1986). During an asthma attack, bronchospasm of the upper and lower airways causes decreased inspiratory and expiratory flow rates (Pride, 1983). Although expiration is ordinarily passive, an increased airway resistance requires the expiratory muscles to perform active work. This increase in active work by the respiratory muscles can create an oxygen debt, lactic acid build-up, fatigue and eventual failure of these muscles.

Conventionally, airway smooth muscle contraction has been measured two ways: isometric or isotonic. In an isometric contraction, the muscle is pulling on an immovable attachment and therefore, the muscle does not undergo any external shortening, and is a measure of tension developed by the muscle. In an isotonic contraction the muscle is attached to a movable lever and therefore, the muscle can shorten, but the tension in the muscle remains constant. Physiologically, both isotonic and isometric contractions occur *in vivo*; however, due to the effect of muscle length changes on intracellular calcium concentration ($[Ca^{++}]_i$) (Gunst, 1989) I have decided, for technical reasons, to examine only the isometric component using bovine tracheal smooth muscle. This will be discussed in the subsequent section.

3. *Species Differences in Trachealis Muscle Mechanics*

Results from *in vitro* airway smooth muscle contraction/relaxation experiments have been obtained using various animals: cow (Kirkpatrick, 1975), dog (Stephens *et al.*, 1969), and human (Marthan *et al.*, 1987). The structural properties of airway smooth muscle from these three different species were analyzed and all three tissue types were found to be similar (Stephens, 1987 A). Accordingly, though my primary objective was to understand smooth muscle contractile processes in humans, studies on bovine smooth muscle should still provide pertinent and important information.

4. *Calcium and the Contractile Mechanism of Airway Smooth Muscle*

Airway smooth muscle tone, unlike that of striated muscle which is largely controlled by nerve recruitment, is modulated by multiple neurotransmitters and mediators (Coburn and Baron, 1990). These neurotransmitters and mediators, once bound to a receptor, can produce a measurable physiological response by activating one or more second messengers within the cytosol.

The role of calcium in the excitation-contraction coupling mechanism of airway smooth muscle was initially based on striated muscle studies (Squire, 1981). However, there are primary biochemical differences between the two types of muscle such as: 1) smooth muscle does not contain Tropinin (a protein used for binding calcium in striated muscle), and 2) smooth muscle ATP hydrolysis can only occur when the 20-kDa light chains of myosin are phosphorylated (Gerthoffer, 1991; De Lanerolle, 1989 A). In striated muscle, the ATP hydrolysis occurs when actin and the myosin interact, but myosin does not need to be phosphorylated.

To achieve smooth muscle contraction, either by pharmacomechanical coupling (pharmacological agent binding to receptors on plasmalemma) or by electromechanical coupling (depolarization in cells membrane), it is essential that the $[Ca^{++}]_i$ increases (Taylor and Stull, 1988; Lofdahl and Barnes, 1986; Panettieri *et al.*, 1989). This change in $[Ca^{++}]_i$ has been estimated to be from a baseline 0.05μM - 0.35μM to 0.35μM - 1μM during cellular contraction (Kotlikoff *et al.*, 1987; Taylor and Stull, 1988; Taylor *et al.*, 1989). Gunst, (1989) found that when muscle length changes, either by passive stretching or by pharmacological stimulation, there is a concomitant change in $[Ca^{++}]_i$ (measured using aequorin chemiluminescence). This implies that to study the pharmacological effects on changes in $[Ca^{++}]_i$, the length of the tissue must be kept constant. It should be noted that, because aequorin chemiluminescence is not a calculated ratio of bound and unbound protein the change in $[Ca^{++}]_i$ could be caused by a change in dye concentration via morphological changes in the cellular conformation.

4.1 *Mechanisms to Increase $[Ca^{++}]_i$*

There are two pathways to increase the $[Ca^{++}]_i$: 1) by activating channels found within the plasmalemma membrane allowing extracellular calcium ions influx into the myoplasm, or 2) by releasing stored calcium ions from the sarcoplasmic reticulum (SR) found within the myoplasm (Sommerville and Hartshorne, 1986). For the first pathway, i.e. calcium entry into the myoplasm via calcium channels, Rodger and Small, (1991) described these calcium channels as being voltage operated (VOC), receptor operated (ROC), or leak (stretch) channels. These calcium channels allow entry of calcium ions from the extracellular space into the myoplasm.

4.1.1 *Voltage Operated Channels*

The VOC are activated when the plasma membrane of the cell is depolarized by, for example, high KCl or tetraethylammonium (TEA) (Rodger, 1987). These channels are categorized as L or T type channels (Triggle *et al.*, 1989). The L type channels are characterized by a large conductance, slow inactivation and are very sensitive to dihydropyridines, whereas, the T type channels are characterized by activation at a more negative membrane potential, rapid inactivation and are relatively insensitive to dihydropyridines. The amount of calcium entering into the myoplasm through the L and T type channels (the calcium conductance of the VOC) is directly proportional to the potential difference across the cell membrane (Tsien, 1983). Activated VOC's can be blocked by specific calcium channel blockers (Verapamil, Nifedipine) (Triggle *et al.*, 1989). Lofdahl and Barnes, (1986) found nifedipine to be the most potent of these blockers on airway smooth muscle tissue.

4.1.2 *Receptor Operated Channels*

The ROC, are controlled by a receptor for a stimulant substance (Bolton 1979), but the existence of ROC in airway smooth muscle has been debated, due the fact that selective inhibitors for these proposed channels in airway smooth muscle have not yet been found (Rodger and Small, 1991).

4.1.3 *Leak Channels*

A small quantity of calcium enters into the myoplasm through leak channels by a passive process. This leak process is caused by the large calcium concentration gradient between the intracellular space and the extracellular medium. However, this passive leak of calcium into the cell does not appear to cause activation of the contractile filaments since the leakage only occurs when the cells are in the resting state (Rodger and Small, 1991).

4.2 *$[Ca^{++}]_i$ Release from Myoplasmic Stores*

The other mechanism for increasing $[Ca^{++}]_i$ is by release from myoplasmic stores, the SR (Summerville and Hartshorne, 1986; De Lanerolle, 1989 B; Rodger and Small 1991; Kirkpatrick, 1975). This pathway was discovered from experiments studying the effects of contractile agonists on airway smooth muscle which was bathed in low calcium Krebs-Henseleit, or by using dihydropyridines which block the L-type channels (Kirkpatrick, 1975; Fukui *et al.*, 1989; Marthan *et al.*, 1987). From their studies the authors suggested that $[Ca^{++}]_i$ increased and caused contraction secondary to the release from myoplasmic stores since extracellular calcium was largely prevented from entering the cells. Also, Coburn and Baron, (1990) found that the myoplasmic stores, such as the SR, can only be activated by pharmacomechanical coupling.

4.2.1 *Myoplasmic Stores and IP3*

The second messenger, inositol-1,4,5,-trisphosphate (IP_3) has been proposed as one mechanism for calcium release from the SR (Chilvers and Nahorski, 1991; Coburn and Baron, 1990). Rapid intracellular IP_3 accumulation has been demonstrated after contractile agonist stimulation of airway smooth muscle (Langlands *et al.*, 1989; Chilvers *et al.*, 1989; Hall *et al.* 1989). This mechanism operates as follows: once the receptor on the plasma membrane is activated, phosphotidylinositol 4,5-bisphosphate (PIP_2) undergoes hydrolysis mediated by a G protein. This transmembrane signalling activates an inositol phospholipid-specific phospholipase C (PLC) enzyme, which cleaves the PIP_2 to form IP_3 and diacylglycerol, both of which accumulate in the myoplasm (Coburn and Baron, 1990). The increase of IP_3 directly activates a channel on the SR membrane which allows calcium to leak into the myoplasm. This channel remains open as long as IP_3 is bound to the SR binding site (Ghosh *et al.*, 1988).

The mechanisms discussed above for increasing and decreasing $[Ca^{++}]_i$ in the myoplasm is illustrated in figure 1.2.

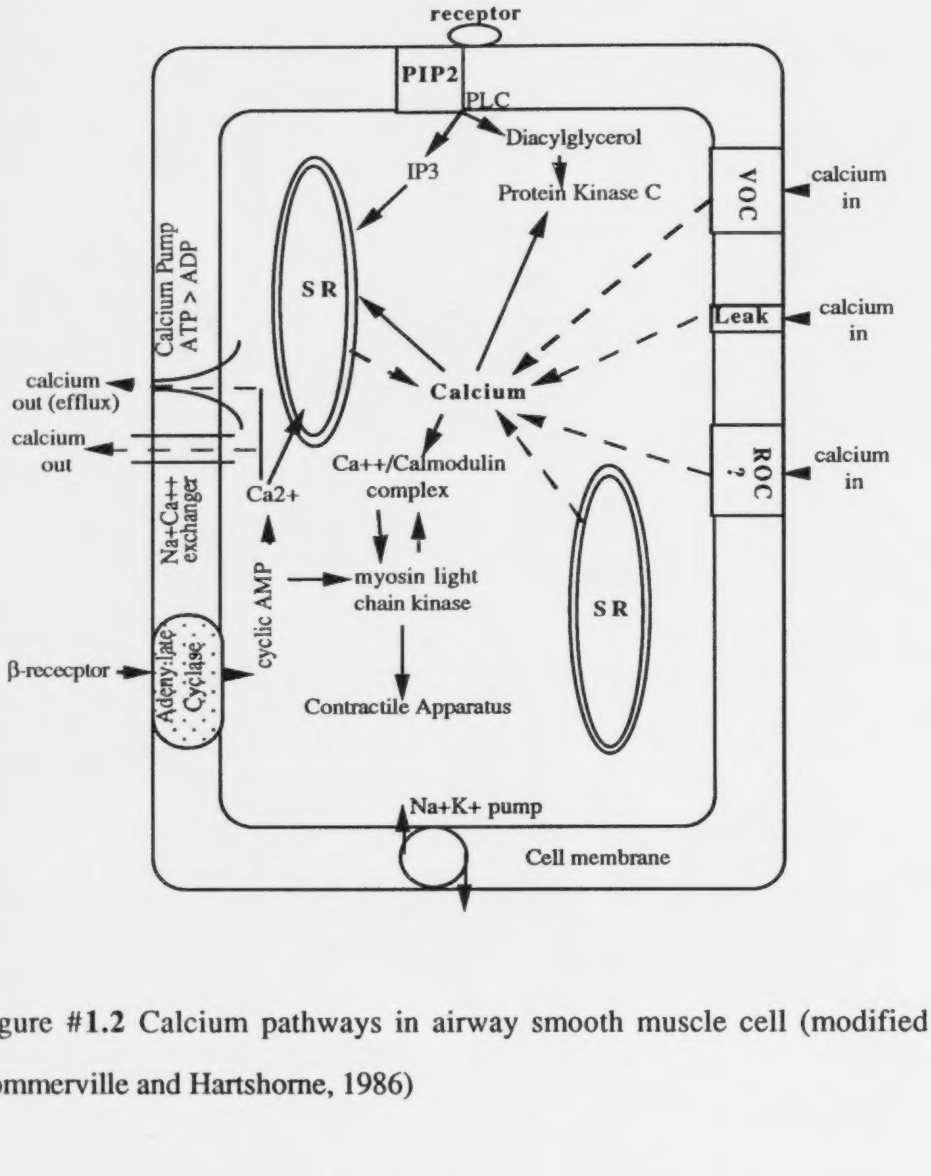
receptor
PIP2
PLC
Diacylglycerol
IP3
Protein Kinase C
VOC
calcium in
S R
Calcium Pump ATP > ADP
Leak
calcium in
calcium out (efflux)
Calcium
calcium out
Na+Ca++ exchanger
Ca2+
Ca++/Calmodulin complex
ROC ?
calcium in
cyclic AMP
myosin light chain kinase
S R
β-rececptor
Adenylate Cyclase
Contractile Apparatus
Na+K+ pump
Cell membrane

Figure #**1.2** Calcium pathways in airway smooth muscle cell (modified from Sommerville and Hartshorne, 1986)

4.3 *Mechanisms to Decrease $[Ca^{++}]_i$*

Because high $[Ca^{++}]_i$ over an extended period of time is toxic to the cell, the calcium is removed from the myoplasm via two mechanisms, 1) via an ATP-driven calcium pump or 2) by a Na^+/Ca^{++} exchanger. The Na^+/Ca^{++} exchanger is a low-affinity, high-capacity system which extrudes calcium whenever the myoplasmic concentration is in the micromolar range. This mechanism has been demonstrated in bovine tracheal smooth muscle (Slaughter *et al.*, 1987). The ATP-driven calcium pump is a high-affinity low-capacity system which has been described to maintain the calcium level at rest (DiPolo and Beauge, 1990).

4.4 *$[Ca^{++}]_i$ and PKC*

Another mechanism for controlling intracellular calcium levels was proposed by Rasmussen *et al.*, (1987) and it involves activation of a phospholipid-dependent protein (protein kinase C, (PKC)). PKC activation is critically dependant on $[Ca^{++}]_i$ and diacylglycerol concentration. The diacylglycerol synthesized via the hydrolysis of PIP_2 causes a translocation of PKC from the cytosol to the endoplasmic face of the plasmalemma. This translocation increases the affinity of PKC for calcium, thus lending itself to modulating the $[Ca^{++}]_i$ during the sustained phase of smooth muscle contraction. It should be noted that the precise physiological function of PKC is not completely understood and Nishizuka, (1988) found that there are several discrete sub-species of PKC which can play a role in the contractile process.

5. *Mechanism of Muscle Contraction*

Once there is a rise in free intracellular calcium, the calcium is available for binding to a specific myoplasmic binding protein (calmodulin) (Cheung, 1980).

The calcium/calmodulin interacts with myosin light chain kinase causing the kinase to phosphorylate myosin at the two 20-kDa light chains. In turn, there is an increase in actinomyosin ATPase which results in cross-bridge cycling (Cheung, 1980; De Lanerolle, 1989). This activated calcium calmodulin pathway is depicted in figure 1.3.

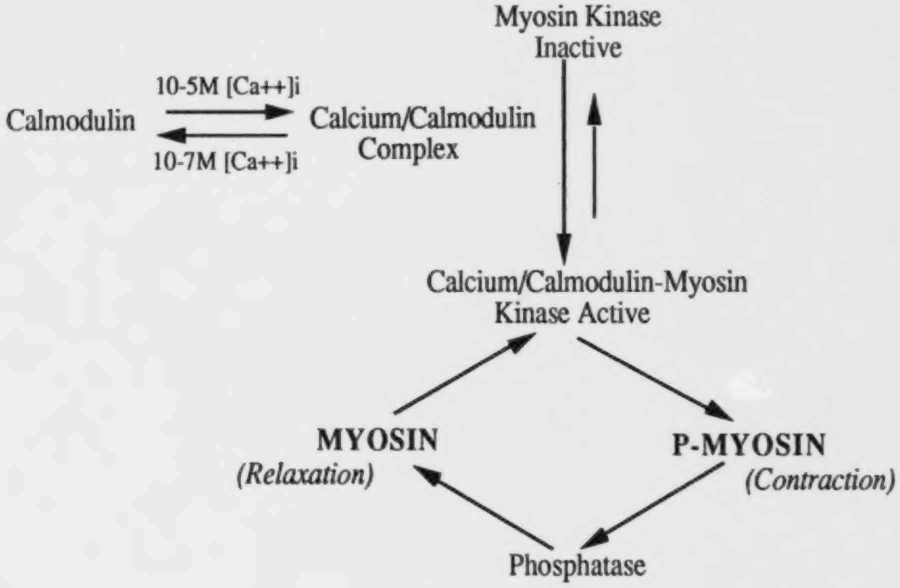
Myosin Kinase
Inactive
10-5M [Ca++]i
Calmodulin
Calcium/Calmodulin
Complex
10-7M [Ca++]i
Calcium/Calmodulin-Myosin
Kinase Active
MYOSIN
(Relaxation)
P-MYOSIN
(Contraction)
Phosphatase

Figure #**1.3** Calcium calmodulin activation (modified from De Lanerolle, 1989 B).

6. *Maintenance of Isometric Tension*

The relationship between the $[Ca^{++}]_i$ and isometric contraction in smooth muscle up to now has probably been oversimplified: an increase in $[Ca^{++}]_i$ will give rise to smooth muscle contraction. To date, it is unclear if increased $[Ca^{++}]_i$ is required to maintain the isometric tension of smooth muscle. Gunst and

Bandyopadhyay, (1989); Aksoy *et al.*, (1983); Karaki, (1989); Ozaki *et al.*, (1990) have all noted that the increase in $[Ca^{++}]_i$ is transient, but the smooth muscle maintains its force when stimulated with certain pharmacological agents. In acetylcholine or carbachol exposure, this transient $[Ca^{++}]_i$ increase is represented by a rapid rise followed by a decline to just above baseline (Gunst and Bandyopadhyay, 1989). It should be noted that the magnitude of change in $[Ca^{++}]_i$ depends on the tissue type, the experimental temperature, the agonist used and its concentration (Gunst, 1989; Rodger and Small, 1990).

An explanation of how tension is maintained during low $[Ca^{++}]_i$ can be explained by the work of Dillon *et al.*, (1981). They examined the phosphorylation of myosin in both relaxed and contracted vascular smooth muscle. They found myosin phosphorylation, like the changes in $[Ca^{++}]_i$, was transient (it increased initially when the muscle contracted, but then fell back to baseline while tension was maintained). These findings were best explained by a new type of cross-bridge: the latch bridge.

6.1 *Latch Bridge*

A latch bridge mechanism initially has cross-bridge cycling (applying the basic function: increase in $[Ca^{++}]_i$ increases the tension) then the cycling slows down and the cross-bridges form a latch or attachment to each other. This attachment maintains the force during the tonic component of the contraction, uses less ATP and therefore requires less activator calcium. De Lanerolle, (1989 B); Rodger and Small, (1991) also applied this latch-bridge theory to airway smooth muscle contraction.

6.2 *PKC*

Rasmussen, (1990) proposed a theory, which was previously mentioned, on how tension is maintained at approximate baseline $[Ca^{++}]_i$ values. Because the myosin light-chain kinase is indirectly affected by the decrease in $[Ca^{++}]_i$ (refer to figure 1.3), he proposed that a calcium-independent myosin light-chain kinase exists. This kinase, which can be phosphorylated by PKC, could therefore keep the myosin light-chains phosphorylated and maintain the contractile machinery during sustained tension.

6.3 *Caldesmon and Calponin*

Gerthoffer, (1991) suggested that there may be another calcium regulatory system which can act on cross-bridge cycling in airway smooth muscle. It involves the calcium calmodulin pathway combined with two other regulatory proteins, caldesmon and calponin, which are found in airway smooth muscle. These two proteins, once phosphorylated, can relieve an inhibition of actomyosin ATPase which occurs normally with resultant increased cross-bridge cycling rate and force production.

7. *$[Ca^{++}]i$ Measurement in Airway Smooth Muscle Tissue*

As mentioned earlier, the level of $[Ca^{++}]_i$ is linked to the contractile mechanism; therefore, an accurate technique to monitor $[Ca^{++}]_i$, would provide useful information. To date, monitoring $[Ca^{++}]_i$ has been accomplished using: calcium selective microelectrodes (Ashley and Campbell, 1979), aequorin (a bioluminescent protein) chemiluminescence (Gunst, 1989; Tukawa *et al.*, 1987), radioactive labelled calcium (Scheid and Fay, 1984), and fluorescent calcium indicators (Ozaki *et al.*, 1990; Kao *et al.*., 1989). Each one of the techniques is useful for specific applications.

7.1 *Calcium Microelectrodes*

In the study of airway smooth muscle contraction, measurement of $[Ca^{++}]_i$ using calcium microelectrodes is not suitable because they cannot follow the rapid calcium fluxes involved in this physiological response and besides, their need for simultaneous membrane potential measurements make them technically difficult to use (Tsien, 1988).

7.2 *Aequorin Chemiluminescence*

The results obtained using aequorin chemiluminescence on airway smooth muscle tissue strip preparations are conflicting (Gunst and Bandyopadhyay, 1989; Tukawa *et al.*, 1987). When isoproterenol was applied to the muscle strip, Gunst and Bandyopadhyay, (1989) found baseline $[Ca^{++}]_i$ values measured by chemiluminescence did not increase whereas, Tukawa *et al.*, (1987) found an increase in $[Ca^{++}]_i$. This difference in results could be caused by different animal tissues or different temperatures during experimentation (Gunst and Bandyopadhyay, 1989), or it could indicate that the aequorin chemiluminescence technique produces variable results and therefore it is not suitable for measuring changes in $[Ca^{++}]_i$ in airway smooth muscle. The results from Tukawa *et al.*, (1987), where isoproterenol increased $[Ca^{++}]_i$, are not supported by the electrophysiological studies on airway smooth muscle cells (Fujiwara *et al.*, 1988). Fujiwara *et al.*, (1988) demonstrated that procaterol, a ß-adrenoceptor agonist, caused the cells to hyperpolarize, reducing the probability of opening of calcium VOC and Marthan *et al.*, (1989) stated that calcium influx and $[Ca^{++}]_i$ under these circumstances should have been reduced rather than increased as suggested by Tukawa *et al.*, (1987).

The discrepancies in the literature on $[Ca^{++}]_i$ changes using aequorin chemiluminescence and the problems associated with using calcium microelectrodes left fluorescent calcium indicators as the only potentially useful technique. Fura II has been used for $[Ca^{++}]_i$ measurement in airway smooth muscle cell preparations (Felbel *et al.*, 1988), and to study $[Ca^{++}]_i$ changes in airway smooth muscle whole tissue preparations (Ozaki *et al.*, 1990). Fluo-3 is a recently developed fluorescent calcium indicator (Minta *et al.*, 1989) which has been found useful for measuring $[Ca^{++}]_i$ in T-lymphocytes, fibroblasts, platelets and neutrophils (Kao *et al.*, 1989; Merritt *et al.*, 1990).

7.3 *Fluorescent Indicators*

When choosing a fluorescent indicator, one must consider all characteristics of the dye. In my case, I required an indicator to measure $[Ca^{++}]_i$ while simultaneously measuring isometric tension in bovine tracheal smooth muscle tissue preparations. Fura II, when used in airway smooth muscle cells or other cell types, does show reproducibility in $[Ca^{++}]_i$ measurement, but this indicator has other major problems when applied to whole tissue preparations. Ozaki *et al.*, (1987) and Sato *et al.*, (1988) found that nicotinamide adenine dinucleotide (NADH) has similar excitation and emission characteristics to myoplasmic fura-2 dye and that the presence of NADH in tissue preparations causes excessive fluorescence at 340 nm while underestimating fluorescence at 380 nm. This NADH affect can cause large errors in the measurement of $[Ca^{++}]_i$. In addition to the NADH interference, the fura II dye excitation wavelengths are within the UV range, and UV light has been shown to cause cellular damage (Merritt *et al.*, 1990; Minta *et al.*, 1989; Williams and Fay, 1986). Still another major problem with fura-II is that the K_d (dissociation constant) increases from 2.5 to 4 fold in some cells due to binding with certain proteins (Wier *et al.*, 1987; Karaki, 1989) and therefore quantitative measurement of $[Ca^{++}]_i$ is difficult or impossible. A fluorescent probe for $[Ca^{++}]_i$, such as fluo-3, which is not affected by other intracellular compounds and which has a fluorescent wavelength outside the UV range seemed to be more desirable for my proposed application.

7.3.1 *Fluo-3 as a Calcium indicator*

Fluo-3 is composed of a calcium selective binding site (an EGTA complex which is similar to other fluorescent indicators) and a distinct fluorophore side chain, (the AM ester derivative has the COO^- groups of fluo-3 replaced by $COOCH_2OCOCH_3$, see molecular structure in figure 1.4) (Tsien, 1988). Fluo-3 operates in the following manner: once the free calcium binds to fluo-3, the electrons on the amino nitrogen are diverted away from the rest of the conjugated system which mimics the disconnection of the nitrogen substituents from the fluorophore. This gives rise to a large spectral change (increase in fluorescent intensity) (Tsien, 1988).

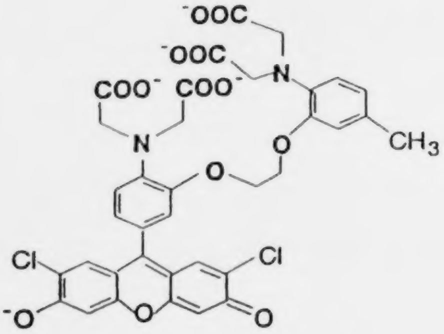

Figure **#1.4** Molecular structure of fluo-3 salt (according to Tsien, 1988).

The fluorescent properties of Fluo-3 make it a potentially useful indicator for $[Ca^{++}]i$ measurement in whole tissue preparations even though its has been primarily used for cellular preparations. Fluo-3 has a single excitation wavelength (506 nm), which is outside the UV range, and at this wavelength NADH does not affect the fluorescent characteristics of this dye. The emission wavelength for fluo-3 is 526 nm. Fluo-3 is almost nonfluorescent as a free dye (when in a calcium free solution), but once converted to the calcium complex within cells, fluo-3 undergoes an approximate 40 fold enhancement of fluorescence (Minta *et al.*, 1989). Another useful characteristic of this dye is its calcium affinity. Fluo-3 has a weaker calcium affinity (a larger dissociation constant (Kd~860 nM at 37 °C) than that of fura-2 (224 nM at 37 °C) (Merritt *et al.*, 1990), which makes it an indicator with increased resolution at both nanomolar and higher levels of $[Ca^{++}]_i$ (Minta *et al.*, 1989). This larger range of calcium measurement is desirable when completing a fluorescent maxima and fluorescent minima calibration curve. The possibility of Kd changing once fluo-3 is in the cytosol has been studied using lymphocytes (Kao *et al.*, 1989) and it was found that no significant change in the Kd occurred after loading with Fluo-3 AM. Therefore, fluo-3 seems to be a good agent for quantitative analysis of $[Ca^{++}]_i$. An undesirable characteristic of fluo-3 is its inability to shift the excitation or emission wavelengths when bound to calcium which compensates for dye concentration changes and therefore, the $[Ca^{++}]_i$ measurement is not a calculated ratio between the amplitudes of the two wavelengths (bound and unbound dye), such as for fura-II and indol-1. However, fluo-3 has many desirable properties and because of these I chose this fluorescent indicator to measure $[Ca^{++}]_i$ in bovine trachealis muscle tissue.

8. *Inflammatory Mediators*

Histamine is a known agonist for smooth muscle contraction, and was used extensively in this thesis. Hence, I have provided an in depth review of its properties. It is a 2-(4-imidazolyl) ethylamine, and its structure is shown in figure 1.5 (Rang and Dale, 1987). In the *in vivo* situation its concentration is highest in the gastrointestinal tract, lung, and skin. It is synthesized from histidine to histamine via L-histidine decarboxylase, and stored in granules within mast cells and basophils (Rang and Dale, 1989).

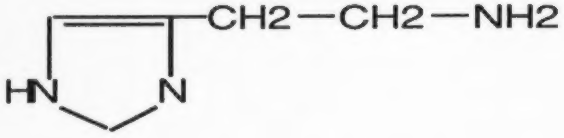

Figure #1.5 Structure of histamine

The possible role it plays in the pathogenesis of asthma is twofold: histamine causes bronchoconstriction, both in the lower and upper airways (Barnes, 1989), and it can cause inflammation and edema in the airway walls (Cooper *et al.*, 1989; Cavanah and Casale, 1987). Cooper *et al.*, (1989) have shown that the release of histamine can occur rapidly in normal bronchi after a non antigenic stimulus, and that this release induces bronchoconstriction *in vivo*. Histamine, when added to the bathing medium, has been shown to isometrically contract bovine (Kirkpatrick, 1975), guinea pig (Fukui *et al.*, 1989) and canine (Katsuyama *et al.*, 1990) tracheal smooth muscle.

The mechanism by which histamine causes contraction of airway smooth muscle is not clearly understood. The proposed theory is that histamine acts directly through the membrane bound H1 receptor (Ash and Schild, 1966; Douglas, 1985) causing (via the mobilization of calcium from intracellular stores) an increase in $[Ca^{++}]_i$ which activates the contractile filaments (Takuwa *et al.*, 1987; Kotlikoff *et al.*, 1987). This elevation in $[Ca^{++}]_i$ has been associated with the myoplasmic elevation of the second messenger 'inositol 1, 4, 5-trisphosphate' (IP_3) (Barnes *et al.*, 1986; Hall and Hill, 1988). The maintenance of force during a histamine-induced contraction has been linked to the PKC mechanism, which was mentioned earlier (Park and Rasmussen, 1985; Takuwa *et al.*, 1987). Also, the influence of extracellular calcium on bovine tracheal smooth muscle contraction, caused by histamine, was demonstrated by extracellular calcium depletion and calcium channel blocker experiments (Kirkpatrick, 1975). Kirkpatrick, 1975 showed that the rate, height and maintenance of tension were associated to extracellular calcium. One other possible mechanism of histamine-induced smooth muscle contraction is via H1 activation of vagal afferent fibres causing a subsequent release of acetylcholine onto the muscle fibres (Cavanah and Casale, 1987). There are also two other histamine receptor subtypes, H2 and H3. The H2 receptor does not appear to play a role in regulating airway smooth muscle tone (Barnes, 1989), but it has been shown to exist in lung homogenates (Foreman *et al.*, 1985). H3 receptors have been shown to have some indirect affects on smooth muscle tone, in that it has been shown to inhibit both cholinergic neurotransmission and the non adrenergic, non cholinergic, bronchoconstriction, which is mediated by neuropeptides such as neurokinin A (Casale, 1991). Also, the H3 receptor, once stimulated, has been shown to inhibit mast cell mediator release and therefore acts as a self-regulating mechanism (Casale, 1991).

Histamine has also been linked to proliferation of airway smooth muscle (Panettieri *et al.*, 1990); increasing the overall muscle mass which is one of the pathological features associated with asthma (Dunnill *et al.*, 1969).

Three other inflammatory mediators were used in my research: **Leukotriene D_4**

(LTD_4), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and platelet-activating factor (PAF), because these have been shown to cause bronchospasm of the airways *in vivo* (Kern *et al.*, 1986; Denjean *et al.*, 1983; Malo *et al.*, 1982). These inflammatory mediators are found *in vivo* and their mechanisms for contraction in airway smooth muscle have not been defined.

LTD_4 and **$PGF_{2\alpha}$** are lipid compounds. They are synthesized from the metabolism of arachadonic acid by the cyclooxygenase and lipoxygenase pathways (Sigal, 1991), and their synthesis is depicted in figure 1.6. LTD4 is produced by many cell types: mast cells, basophils, monocytes, eosinophils, and macrophages (Abraham and Wanner, 1988) and causes isometric contraction *in vitro* in both human and guinea pig airway smooth muscle (Kohrogi *et al.*, 1985; Advenir *et al.*, 1983). The contractile response caused by LTD4 in human airways is thought to occur by interaction with specific airway receptors and not by production of cyclooxygenase products (Drazen and Austen, 1987).

PGF2α is produced by basophils, neutrophils, eosinophils, macrophages, mast cells, and many other cell types (Abraham and Wanner, 1988), and has been shown to cause contraction in vascular, iris sphincter and airway smooth muscle *in vitro* (Asano *et al.*, 1989; Yousufzai *et al.*, 1989; Douglas and Brink, 1987). Abraham and Wanner, 1988 state that "the cyclooxygenase metabolites may be more important in modulating the airway effects rather than in mediating them.

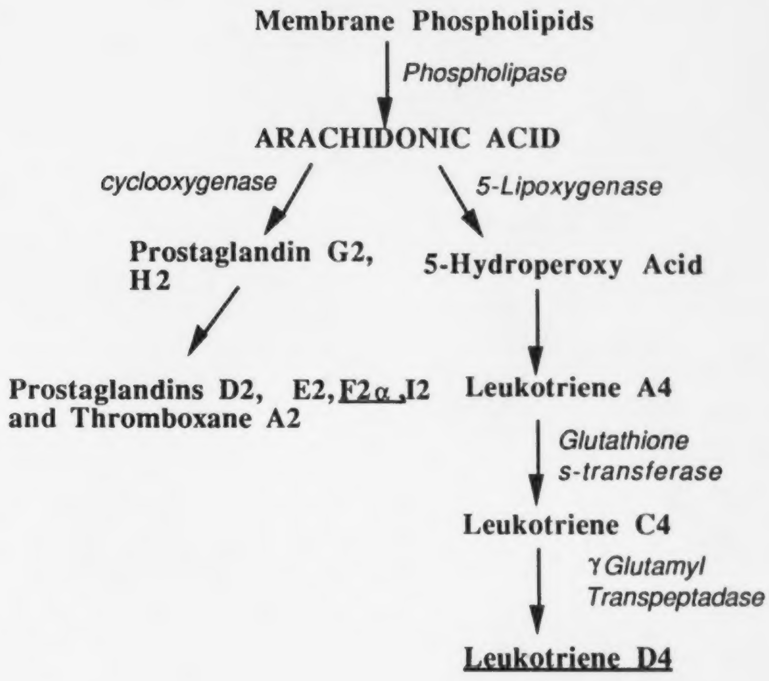
Membrane Phospholipids
Phospholipase
ARACHIDONIC ACID
cyclooxygenase
5-Lipoxygenase
Prostaglandin G2, H2
5-Hydroperoxy Acid
Prostaglandins D2, E2, F2α, I2 and Thromboxane A2
Leukotriene A4
Glutathione s-transferase
Leukotriene C4
γ Glutamyl Transpeptadase
Leukotriene D4

Figure #1.6 Simplified arachadonic acid metabolism (modified from Abraham and Wanner, 1988)

PAF, is a 1-*0*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine compound (Demopoulos *et al.*, 1979), formed by a two step process: 1) deacylating the 1-*0*-alkyl-2-acyl-glycerophosphocholine giving the inactive precursor Lyso-PAF and 2) acetylation of Lyso-PAF by acetyltransferase making the active PAF compound mentioned above. PAF is found in basophils, neutrophils, eosinophils, macrophages, and possibly mast cells (Abraham and Wanner, 1988). The contractile effect of PAF on canine tracheal smooth muscle strips is thought to require mediation by the release of 5-Hydroxytryptamine (serotonin) from platelets (Popovich *et al.*, 1988) and therefore, platelets are felt to be necessary (in the bathing medium) to elicit any PAF-induced contractile response in tracheal and bronchial smooth muscle (Schellenberg *et al.*, 1983; Popovich *et al.*, 1988).

9. ß *Agonist as a Muscle Relaxant*

Because bronchospasm is one of the main features of asthma, the relaxation of airway smooth muscle is paramount for returning the asthmatic patient to his or her normal state. Ahlquist, (1948) showed that adrenaline alleviates bronchospasm and that two types of receptors exist on smooth muscle, α and ß. Using autoradiographic studies, Carstairs *et al.*, (1985) confirmed that human bronchial smooth muscle ß-adrenoceptors are entirely of the $ß_2$ subtype. The selectivity for the $ß_2$-adrenoceptors is important, because this specific receptor causes the biochemical events which mediate relaxation in airway smooth muscle. The differences between α, $ß_1$ and $ß_2$ have led to the development of adrenergic agonists that can preferentially act on $ß_2$ receptors of the airway smooth muscle, with limited stimulation of the $ß_1$ receptor which has profound cardiovascular effects (Meltzer and Kemp 1991). For many years the treatment for asthma has been based on ß-agonist inhalation therapy (Paterson *et al.*, 1979).

9.1 *Salbutamol*

The ß2-agonists resemble the endogenous compound adrenaline, except that they are more selective for the $ß_2$-adrenoceptors (Meltzer and Kemp, 1991).

Salbutamol (trade name Ventolin) was chosen for its relaxant properties, because it is one of the drugs used for controlling asthma, and it is very specific for the ß$_2$-adrenoceptor. The molecular structure of salbutamol is shown in figure 1.7.

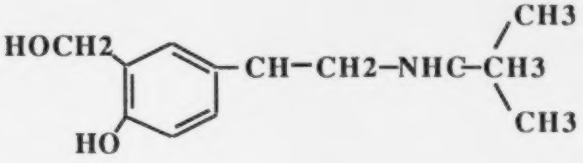

Figure #1.7 Molecular structure of salbutamol (according to Meltzer and Kemp, 1991).

The following is the proposed mechanism for inhaled salbutamol-induced relaxation of airway smooth muscle: After inhalation of salbutamol aerosol the compound binds to ß$_2$-adrenoceptors (Stiles *et al.*, 1984) which are found on the exterior surface of the cell membrane of epithelium, serous glands, and airway smooth muscle (Barnes and Basbaum, 1983). After attaching to the ß$_2$-adrenoceptor, salbutamol causes a reaction of guanyl nucleotide binding stimulator protein (G protein known as Ns or Gs) (Benovic *et al.*, 1985) and activates adenylate cyclase (a single polypeptide glycoprotein (Gilman, 1987)). The Gs protein is a heterotrimer composed of α, ß and γ subunits (Caron, 1985); the α subunit contains the binding site for guanosine 5'-triphosphate (GTP), and the ß and γ subunits act as inhibitors for adenylate cyclase activation (Gilman, 1987). Activation of adenylate cyclase results from binding guanosine diphosphate (GDP) to the α subunit which is later replaced by GTP. Once the GTP is bound to the α subunit the Gs heterotrimer breaks apart (ß and γ subunits are shed) leaving an Gs-alpha-GTP conglomeration (Gilman, 1987). This Gs-alpha-GTP unit then activates the adenylate cyclase pathway. Once activated, the adenylate cyclase combines with other compounds (found intracellularly) to produce adenosine 3', 5', cyclic monophosphate (cyclic AMP), which causes smooth muscle relaxation. The association between adenylate cyclase and cyclic AMP will be discussed in greater detail under the subheading relaxation of airway smooth muscle.

10. *Cyclic Nucleotides and Airway Smooth Muscle*

Cyclic AMP and cyclic 3'-5'-guanosine monophosphate (cyclic GMP) are two main nucleotides which have been examined extensively for their effect on smooth muscle tone. Meltzer and Kemp, (1991) stated that "the parasympathetic limb of the autonomic nervous system is responsible for bronchoconstriction which is mediated by cyclic GMP, and the sympathetic system controls bronchodilation which is mediated by cyclic AMP." This relationship, also known as the *"Ying Yang"* hypothesis (Goldberg *et al.*, 1975), is based on the concept that intracellular cyclic GMP mediates contraction only, whereas, intracellular cyclic AMP mediates relaxation only. This theory is based on results from various types of smooth muscle, but it may not hold for airway smooth muscle. Torphy *et al.*, (1985) added a membrane permeable analog of cyclic GMP (8-bromo-cyclic GMP) to precontracted tracheal smooth muscle strips and discovered that it too, caused relaxation. In addition, Pfitzer *et al.*, (1984) found that adding cyclic GMP to vascular smooth muscle caused a significant relaxation.

Katsuki and Murad, (1976) approached the cyclic nucleotide dilemma by simultaneously measuring intracellular cyclic AMP and cyclic GMP concentrations along with isometric tension in bovine tracheal smooth muscle tissue strips. Their aim was to determine if any correlation existed between the two cyclic nucleotides and isometric tension. They found neither a consistent parallel, or a reciprocal change in cyclic AMP or cyclic GMP levels during contraction or relaxation induced by a variety of agents and therefore, their concentrations are not correlated (Katsuki and Murad, 1976). Andersson *et al.*, (1975) completed studies on various types of smooth muscle, including bovine tracheal smooth muscle, and they found no simple relationship between the contractile state of the muscle and accumulation of either cyclic nucleotides.

By establishing a seemingly independent role between cyclic GMP and cyclic AMP, and determining that there is no correlation between the two, I focused my research on the nucleotide linked to airway smooth muscle relaxation "cyclic AMP".

To help establish a link between airway smooth muscle tone and cyclic AMP concentration, Gold, (1980) stated that "relaxation of many smooth muscles is often associated with increased concentration of cyclic AMP" and "contraction is not generally associated with decreased concentration of cyclic AMP." Rinard *et al.*, (1983) supported Gold's statement by reporting a significant increase in cytoplasmic cyclic AMP concentration when tracheal smooth muscle tissue was preincubated with isoproterenol. Using precontracted bovine tracheal smooth muscle Hall *et al.*, (1989) found that there was a significant and maintained accumulation of cytosolic cyclic AMP when the muscle was relaxed with salbutamol.

An alternate way to increase the cyclic AMP concentration is by inhibiting the breakdown of cyclic AMP. Hall *et al.*, (1989) found significant increases in accumulation of cyclic AMP when bovine tracheal strips were pretreated with isobutyl methylxanthine (IBMX), a non-specific phosphodiesterase inhibitor. Hall *et al.*, (1989) also noted that the effect of phosphodiesterase-inhibition (cyclic AMP increase) on histamine induced inositol phosphate accumulation was saturable.

10.1 *Multidimensional Role of Cyclic AMP on Smooth Muscle Contraction*

To this point I have described how relaxation of smooth muscle is associated with cyclic AMP concentration. There are however, some discrepancies in the literature on cyclic AMP levels during airway smooth muscle contraction. Katsuki and Murad, (1976) simultaneously measured cyclic AMP concentration and isometric tension in bovine tracheal smooth muscle. They found that the concentration of cyclic AMP increased when the muscle strip was stimulated with bronchodilating adrenergic agonists, and also with high concentrations of the bronchoconstrictors histamine, carbachol, and K^+. These results suggest that there is no association between relaxation of airway smooth muscle and cyclic AMP accumulation and/or that there are possibly different functional pools of cyclic AMP in airway smooth muscle (Katsuki and Murad, 1976). Palmer, (1972) noted that histamine-induced contraction of guinea pig lung slices caused an increase in cyclic AMP, and Andersson and Nilsson, (1978) using bovine tracheal smooth muscle showed similar results. The cholinergic agonist, carbamylcholine causes decreased levels of cyclic AMP in bovine tracheal smooth muscle (Andersson and Nilsson 1978), but Murad and Kimura, (1974) found acetylcholine increased cyclic AMP levels in guinea pig tracheal rings. The results using other pharmacologic agonists, such as α adrenergic agonists, are also inconsistent; they either inhibit the increase of cyclic AMP (Triner *et al.*, 1971) or had no effect on the level of cyclic AMP (Murad and Kimura, 1974).

The variations in cyclic AMP concentration described above suggests that more research is needed and the role of cyclic AMP in airway smooth muscle seems multidimensional.

10.2 *Cyclic AMP Synthesis*

Synthesis of cyclic AMP occurs in the cytosol of the cells, near or on the cell membrane (Gold, 1980). Cyclic AMP is formed from adenosine triphosphate (ATP) by a reaction catalyzed by adenylate cyclase. This cyclic AMP formation process has been shown to be endergonic and is accelerated by the subsequent hydrolysis of pyrophosphate (Greengard and Kuo, 1970). Once the cyclic AMP is formed it binds to either a specific protein kinase in airway smooth muscle (Gold, 1980), or to voltage operated calcium channels causing decreased activation of calcium flux in the vascular smooth muscle cells (Meisheri and Van Breeman, 1982), or to dihydropyridine insensitive calcium channels causing increased activation and a calcium flux into airway smooth muscle cells (Takuwa *et al.* 1988). Related to the first proposed cyclic AMP binding, there are two types of soluble cyclic AMP-dependant protein kinases (type I and type II) (Corbin *et al.*, 1975). The variation between the two kinases is the type of receptor subunit (R2) isoenzymes found on the R2C2 complex shown below. Each kinase has two receptor subunits (R2) which bind the cyclic AMP and two catalytic units (C2) (Gold, 1980).

R2C2 (inactive) + 4 cyclic AMP--> 2{R(cyclicAMP)2} + 2C(active)

(Goldie *et al.*, 1991)

After being synthesized, the cyclic AMP molecules bind to the regulatory subunits of the protein kinases "A-kinase" (Adelstein *et al.*, 1981). This complex can then phosphorylate specific proteins setting in motion a variety of biochemical events which result in the relaxation of airway smooth muscle (Gold, 1980); this will be discussed in greater detail below.

The effects of cyclic AMP on calcium channels in airway smooth muscle are not clearly established. As mentioned earlier, Tukawa *et al.*, (1989) proposed that the rise in cyclic AMP causes an increase in $[Ca^{++}]_i$ from activation of a dihydropyridine insensitive calcium channel, whereas Gunst and Bandyopadhyay, (1989) found no change in $[Ca^{++}]_i$ when the trachealis strip was stimulated with isoproterenol.

10.3 *Cyclic AMP Degradation*

The metabolism of cyclic AMP is completed by a specific phosphodiesterase.

There are many different types of phosphodiesterases which act on various isoforms: calcium-calmodulin stimulated phosphodiesterase I (PDE I) (Reeves *et al.*, 1987), cyclic GMP selective phosphodiesterase II (PDE II), cyclic AMP specific phosphodiesterase III and IV (PDE III and PDE IV) (Appleman *et al.*, 1973), and a non-selective phosphodiesterase (PDE).

These phosphodiesterases are found within the cells of the tissue (Gold, 1980) and both PDE III, IV and PDE phosphodiesterases hydrolyse cyclic AMP to the inactive form (adenosine monophosphate) (Appleman *et al.*, 1973). The phosphodiesterase activity was found to be critically modulated by calcium regulatory proteins, and these calcium regulatory proteins effectively increase the soluble, but not the membrane bound, phosphodiesterases (Chang, 1971). The stability of cyclic AMP in the cytosol is determined by the concentration of phosphodiesterase present in the cytosol (Greengard and Kuo, 1970).

10.4 *Cyclic AMP and Airway Smooth Muscle Relaxation*

There are two proposed mechanisms of how 'increased cyclic AMP concentration' mediates relaxation of smooth muscle. One mechanism is by **activation of cyclic AMP-dependant protein kinase** which phosphorylates myosin light chain kinase (MLCK) (Silver and Stull 1982). Once the MLCK is phosphorylated it has a decreased affinity for the calcium/calmodulin complex which decreases the phosphorylation of myosin causing a reduction in actin/myosin interaction (cross bridge cycling) and thus, relaxation of the muscle (Adelstein *et al.*, 1978; Conti *et al.*, 1981; and Nishikawa *et al.*, 1984). The second proposed mechanism for cyclic AMP-induced smooth muscle relaxation involves a **decrease in $[Ca^{++}]_i$**. A decrease in $[Ca^{++}]_i$ has been shown to occur by: a re-uptake of intracellular calcium via the sarcoplasmic reticulum (Itoh *et al.*, 1982), a reduction in calcium influx (Meisheri and Van Breeman, 1982), an increase in calcium efflux (Bulbring and den Hertog, 1980; Scheid *et al.*, 1979; and Van Breeman, 1977).

Measurement of $[Ca^{++}]_i$ when cyclic AMP levels are raised should indicate the relationship between the two second messengers, but it will not show were the calcium is mobilized (extracellularly, S.R.). To establish a better understanding of the mobilization of calcium in relationship to elevated cyclic AMP levels in tracheal smooth muscle the measurement of both intra and extracellular calcium will be compared at various cyclic AMP concentrations. Measurement of extracellular calcium surrounding the trachealis muscle, using fluo-3 pentaamonnium salt (cell impermeant), when relaxed with salbutamol and/or precontracted with histamine is an important experiment, because 1) it is an attempt at looking at small changes in the extracellular calcium concentration and 2) the proposed smooth muscle relaxation experiments describing the calcium pathway were completed on smooth muscle other than airway. Rodger and Small, (1990) reported that "there have, as yet, been no reports of action of ß-adrenoceptor agonists in promoting the extrusion of calcium from airway smooth muscle". However, Scheid *et al.*, (1979) gave a clear explanation of the calcium efflux mechanism based on results obtained using stomach smooth muscle cells: the calcium efflux was caused by ß-adrenoceptor stimulation which increased the activity of cyclic AMP-dependant protein phosphorylation. This cyclic AMP-dependant protein phosphorylation caused the sodium/potassium pump to increase ion movement, thereby indirectly activating the sodium/calcium exchanger to decrease the $[Ca^{++}]_i$. Results obtained from simultaneous measurement of extracellular calcium and isometric tension when combined with results from simultaneous measurement of intracellular calcium and isometric tension would be beneficial in understanding calcium fluxes in airway smooth muscle tissue in different stages of contraction or relaxation.

The contraction of smooth muscle, as stated earlier, is dependant on the free calcium ions within the cytoplasm of the cell (Bolton, 1979; Taylor and Stull, 1988; and Takuwa *et al.*, 1987). Therefore, decreasing intracellular calcium should cause relaxation. DeLanerolle,(1989 A) explained the decreased calcium relaxation mechanism as follows: a decrease in intracellular free calcium to $\simeq 10^{-7}$ M leads to a dissociation of the calcium-calmodulin complex from the myosin light-chain kinase, which results in the inactive form of the enzyme which causes relaxation of the muscle. Figure 1.8 depicts both mechanisms discussed above and how they can cause relaxation.

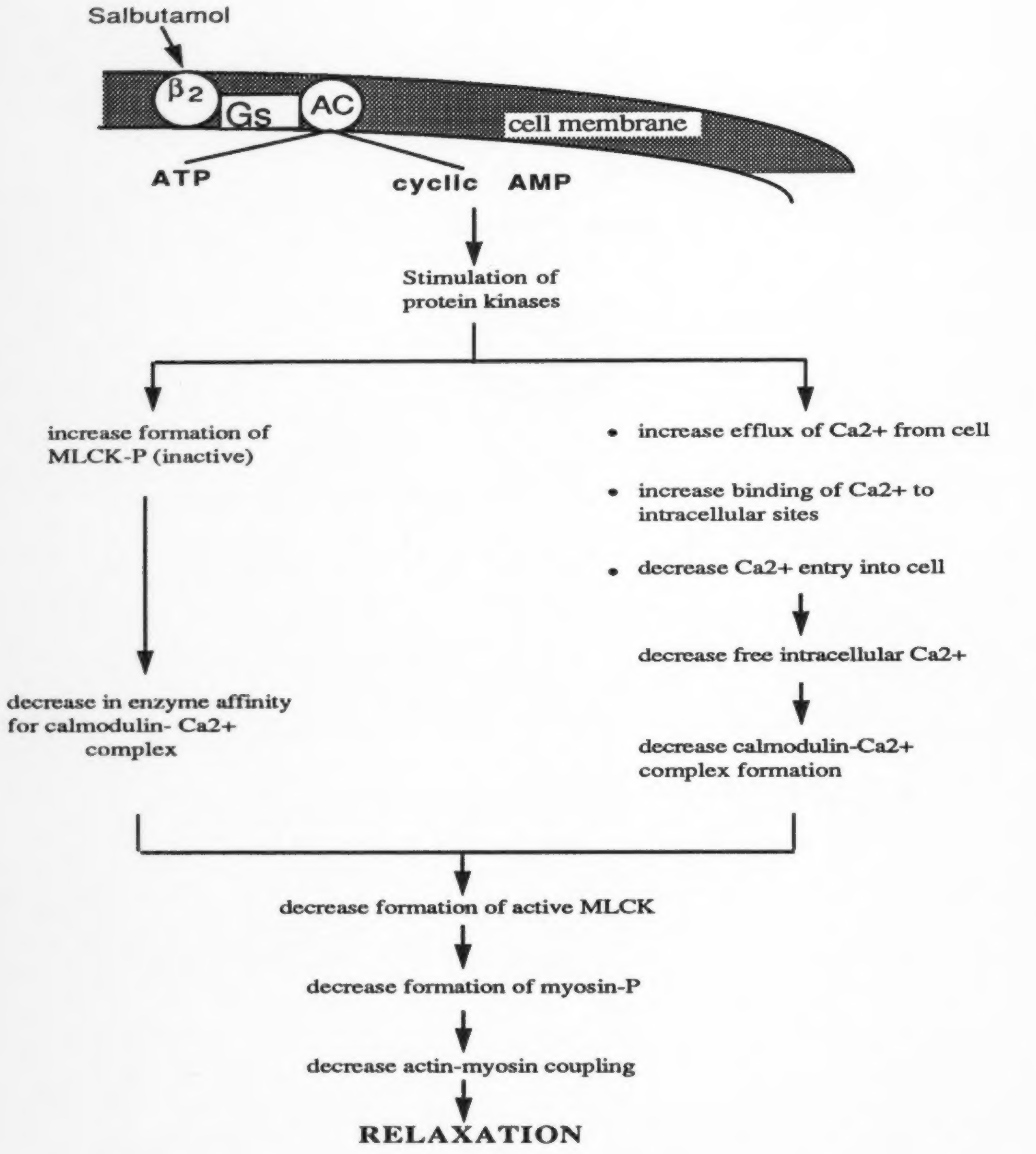
Salbutamol
β2
Gs
AC
cell membrane
ATP
cyclic AMP
Stimulation of protein kinases
increase formation of MLCK-P (inactive)
decrease in enzyme affinity for calmodulin- Ca2+ complex
increase efflux of Ca2+ from cell
increase binding of Ca2+ to intracellular sites
decrease Ca2+ entry into cell
decrease free intracellular Ca2+
decrease calmodulin-Ca2+ complex formation
decrease formation of active MLCK
decrease formation of myosin-P
decrease actin-myosin coupling
RELAXATION

Figure #**1.8** Proposed mechanisms for smooth muscle relaxation. (modified from Goldie *et al.*, 1991)

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Chapter II

Effects of Leukotriene D_4, Prostaglandin $F_{2\alpha}$, Platelet-Activating Factor, or Histamine on Isometric Tension in Bovine Tracheal Smooth Muscle

Introduction

Leukotriene D_4 (LTD_4), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), platelet activating factor (PAF) and histamine have been shown to cause airflow obstruction in humans and other species when administered by inhalation (Kern *et al*., 1986; Denjean *et al*., 1983; Malo *et al*., 1982). These inflammatory mediators are found *in vivo* and their ability to cause isometric contractions in tracheal smooth muscle *in vitro* is not well understood.

Synthesis of Inflammatory Mediators

LTD_4 and $PGF_{2\alpha}$ are lipid compounds synthesized from arachidonic acid by the lipoxygenase and cyclooxygenase pathways, respectively (Sigal, 1991). LTD_4 is produced by mast cells, basophils, monocytes, eosinophils, and macrophages (Abraham and Wanner, 1988). $PGF_{2\alpha}$ is produced by basophils, neutrophils, eosinophils, macrophages, mast cells, and other cell types (Abraham and Wanner, 1988). Furthermore, Abraham and Wanner, (1988) also noted that both eosinophils and neutrophils were found in human bronchoalveolar lavage fluid during the late response of an antigen-induced asthmatic response. PAF is found in basophils, neutrophils, eosinophils, macrophages, and possibly mast cells (Abraham and Wanner, 1988). PAF (1-*0*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine compound (Demopoulos *et al.*, 1979) is formed by a two step process: 1) deacylating the 1-*0*-alkyl-2-acyl-glycerophosphocholine giving the inactive precursor Lyso-PAF and 2) acetylation of Lyso-PAF by acetyltransferase making the active PAF. Histamine a 2-(4-imidazolyl) ethylamine, is synthesized from histidine to histamine via L-histidine decarboxylase and is stored in granules within mast cells and basophils (Rang and Dale, 1989).

Contractile Responses Induced by LTD_4, $PGF_{2\alpha}$, PAF and Histamine

LTD_4 has been shown to induce isometric contraction of human bronchial airway smooth muscle *in vitro* (Kohrogi, 1985). In the equine respiratory tract, LTD_4 stimulation only caused a contractile response in parenchyma strips at $\simeq 1$ μM. Furthermore, the trachealis muscle strips showed no contractile response to either low (0.3 μM) or high (450 μM) concentrations (Doucet *et al.*, 1990). The above findings suggest that either peripheral airway smooth muscle has a different sensitivity to LTD_4 than does trachealis muscle, or that parenchyma tissue, because it is composed of a heterogeneous mixture of cells (one being vascular smooth muscle cells) could give inconclusive results regarding airway smooth muscle contractility.

$PGF_{2\alpha}$ has been shown to cause a contractile response that is tachyphylactic in guinea-pig tracheal smooth muscle *in vitro* (Douglas and Brink, 1987). In addition, the $PGF_{2\alpha}$-induced tachyphylaxis could be reversed by indomethacin, a known cyclooxygenase inhibitor. These findings have not been reported in bovine tracheal smooth muscle.

The contraction of canine tracheal smooth muscle strips by PAF were shown to be tachyphylactic and mediated by the platelets surrounding the smooth muscle, which must be present in the bathing medium (Popovich *et al.*, 1988).

The finding was similarly reported in human bronchial strips (Schellenberg *et al*., 1983) and in guinea-pig tracheal muscle preparations (Morley *et al.*, 1989). Popovich *et al*., (1988) concluded that the PAF-induced contraction is mediated by 5-Hydroxytryptamine (5-HT) which is released from platelets in response to PAF stimulation and that the tachyphylaxis is due to a depletion of 5-HT from the platelets.

Histamine, when added to the bathing medium, has been shown to isometrically contract bovine (Kirkpatrick, 1975), guinea pig (Fukui *et al.*, 1989) and canine (Katsuyama *et al.*, 1990) tracheal smooth muscle. The proposed mechanism is through the membrane bound H1 receptor (Ash and Schild, 1966; Douglas, 1985) which causes (via the mobilization of calcium from intracellular stores) an increase in $[Ca^{++}]_i$ which activates the contractile filaments (Takuwa *et al.*, 1987; Kotlikoff *et al.*, 1987). This elevation in $[Ca^{++}]_i$ has been associated with the myoplasmic elevation of the second messenger 'inositol 1, 4, 5-trisphosphate' (IP_3) (Barnes *et al.*, 1986; Hall and Hill, 1988). The maintenance of force during a histamine-induced contraction has been linked to the PKC mechanism (Park and Rasmussen, 1985; Takuwa *et al.*, 1987). Also, the influence of extracellular calcium on bovine tracheal smooth muscle contraction, caused by histamine, was demonstrated by extracellular calcium depletion and calcium channel blocker experiments (Kirkpatrick, 1975). Kirkpatrick, (1975) showed that the height and maintenance of tension were associated to extracellular calcium.

The roles of these inflammatory mediators in the pathogenesis of airway constriction, though not fully defined, are likely important. As their actions on airway smooth muscle have yet been well understood, and I wished to explore the feasibility of looking at their mechanisms of action; I have decided to firstly determine if I could obtain reproducible isometric contractile responses with these compounds.

In this study using bovine tracheal smooth muscle, my **objectives** were to determine: 1) if LTD_4 causes reproducible isometric contraction, 2) if $PGF_{2\alpha}$ will cause a dose-dependant reproducible response, 3) if PAF causes isometric contraction in the absence of platelet addition to the bathing medium, and 4) if histamine causes a) a dose-dependent response, b) tachyphylaxis, and c) a tension variation when the tissue strip is maintained in low extracellular calcium.

Materials and Methods

Prostaglandin $F_{2\alpha}$, leukotriene D_4, cyproheptadine hydrochloride were purchased from Sigma (St. Louis, MO. USA). Platelet-activating factor (1-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL. USA). Krebs-Henseleit (KH) buffer for these experiments contained (in mM) 113 NaCl, 4.8 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, and 5.5 glucose. Modified Krebs-Henseleit buffer (MKH) for these experiments contained (in mM) 113 NaCl, 4.8 KCl, 0.1 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, 5.5 glucose and 0.14 EGTA (Stockbridge, 1987).

Smooth Muscle Isolation

All tracheas were obtained fresh from a local abattoir. Ten centimetres of midcervical trachea was excised from 1-2 year old steers during exsanguination, and placed in KH solution at 4 °C during transportation to the laboratory (approximately 30 minutes). While submersed in KH solution the epithelial layer was removed and a piece of trachealis muscle approximately 1 mm wide x 2 mm thick x 10 mm long was tied at both ends using 5-0 surgical silk, and then excised from the connective tissue sheath. The wet weight of the muscle strips varied between 20 to 30 mg and were composed largely of smooth muscle cells (figure 2.1).

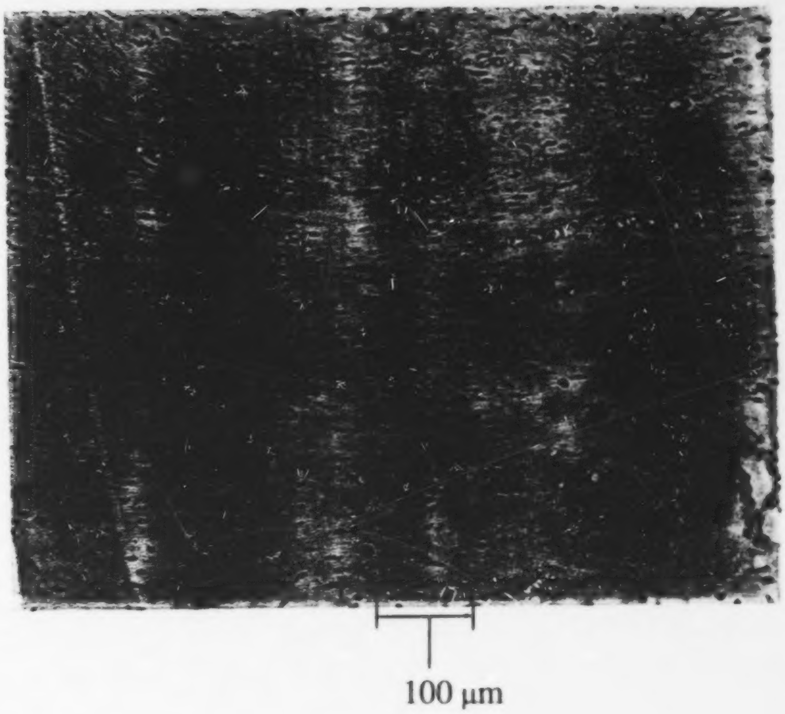

Figure # 2.1 Longitudinal cross section of bovine tracheal smooth muscle at L_o. (Section was stained with toluidine blue x 160)

Isometric Tension Measurements

Trachealis muscle strips were mounted in tissue baths as depicted in figure 2.2 The tissue strips were bathed in KH solution which was oxygenated with 95% O_2 - 5%CO_2 (carbogen) with the bicarbonate buffer maintaining the pH at 7.4. The temperature was maintained at 37 °C.

To establish the active resting tension (L_o), which is the minimum tension that will generate the maximum response when stimulated electrically or via contractile agonist, for each individual muscle strip I used a standard procedure similar to that described by Gunst, (1989). This procedure consisted of electric field stimulation (A.C. source of 14-V, 60-Hz for 10secs) every 5 minutes, and adjusting the muscle length periodically to generate the maximum isometric tension (Po).

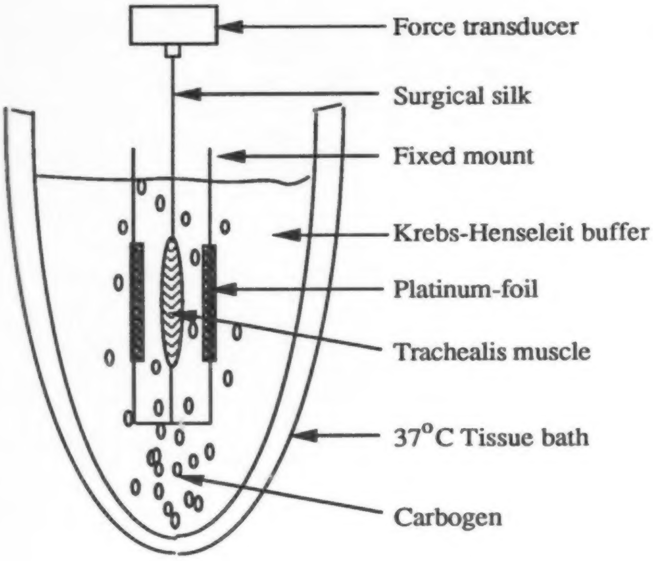
Force transducer
Surgical silk
Fixed mount
Krebs-Henseleit buffer
Platinum-foil
Trachealis muscle
37°C Tissue bath
Carbogen

Figure # 2.2 Tissue, and Bath Assembly.

The equilibration process took approximately 90 minutes, after which the Lo was measured.

The experiments (8 in total) consisted of 4 to 6 isometric contractions either as cumulative or individual dose responses using one of the following compounds: LTD_4 (0.01, 0.1, 1 and 10 μM), PAF (50 μM), $PGF_{2\alpha}$ (0.01, 0.1, 0.5 1, 10, 50 and 100 μM), acetylcholine (ACh) (5 μM) or histamine (0.001, 0.01, 0.1, 1, 3.5, 10 and 100 μM) as the contractile agents. Histamine-induced isometric tension was evaluated for the possibility of tachyphylaxis and during exposure to low extracellular calcium (MKH solution ~ 100 nM (Stockbridge, 1987)). To examine the possibility of tachyphylaxis the tissue strips were contracted with 4 identical doses of histamine at ED50, where, between each dose the muscle strip was rinsed 2 times and the L_o reestablish. For low extracellular calcium experiments the tissue and tissue bath were first rinsed twice with MKH solution (~ 1 minute), and after 2 minutes the tissue was exposed to histamine (ED50) in the MKH solution.

The isometric tension-time curves generated by each muscle strip was recorded continuously for approximately 1.5 minutes prior to drug administration and for 12 minutes thereafter. For individual dose experiments, following the first dose, the tissue strip was rinsed twice in KH solution and L_o reestablished before the next dose of the compound was tested.

Muscle tension was recorded on a chart recorder (GOULD 2600 S) and digitized and stored on an IBM computer. The tension was initially recorded as g/sec and then converted to $mN/mm^2/sec$ incorporating the cross sectional area (CSA) of the tissue strip. Studies were acetylcholine (ACh) or two inflammatory mediators were administered, the histamine or acetylcholine peak response was considered 100%, and the responses caused by other compounds were expressed as a percentage of the peak response. At the completion of the studies, each muscle strip was rinsed and removed from the apparatus. The muscle strips were then weighed and the CSA was calculated: CSA (mm^2) = [density (mg/mm^3) x tissue strip wet weight (mg)] / length at L_o (mm) (Jiang *et al.*, 1991) where the density of the muscle strip was assumed to be 1 mg/mm^3 (Stephens *et al.*, 1984)

Statistical Analysis

Data is presented as mean ± SEM. Comparisons between groups of pharmacological interventions were by one-way ANOVA ($P \leq 0.05$), followed by the Newman-Keuls multiple-comparison test (0.05 critical value).

Results

Effect of LTD_4 on Isometric Tension

Figure 2.3, shows typical isometric tension-time curves caused by LTD_4 stimulation. The isometric tension developed was transient with the tension decreasing to below baseline values after peak tension was obtained (n=2). In figure 2.4, the peak tensions induced by cumulative doses of LTD_4 (0.01, 0.1, 1 and 10 μM) on 4 tissue strips was significantly lower than that produced by stimulation with ACh (5 μM) ($P \leq 0.05$). At 0.1 μM, LTD_4 induced the largest contraction. In figure 2.5 the addition of ACh (5 μM) did not cause an increase in isometric tension when added in the presence of LTD_4. However, in the same tissue, after rinsing with KH solution, the same dose of ACh (5 μM) caused a brisk contraction, the magnitude of which was not diminished.

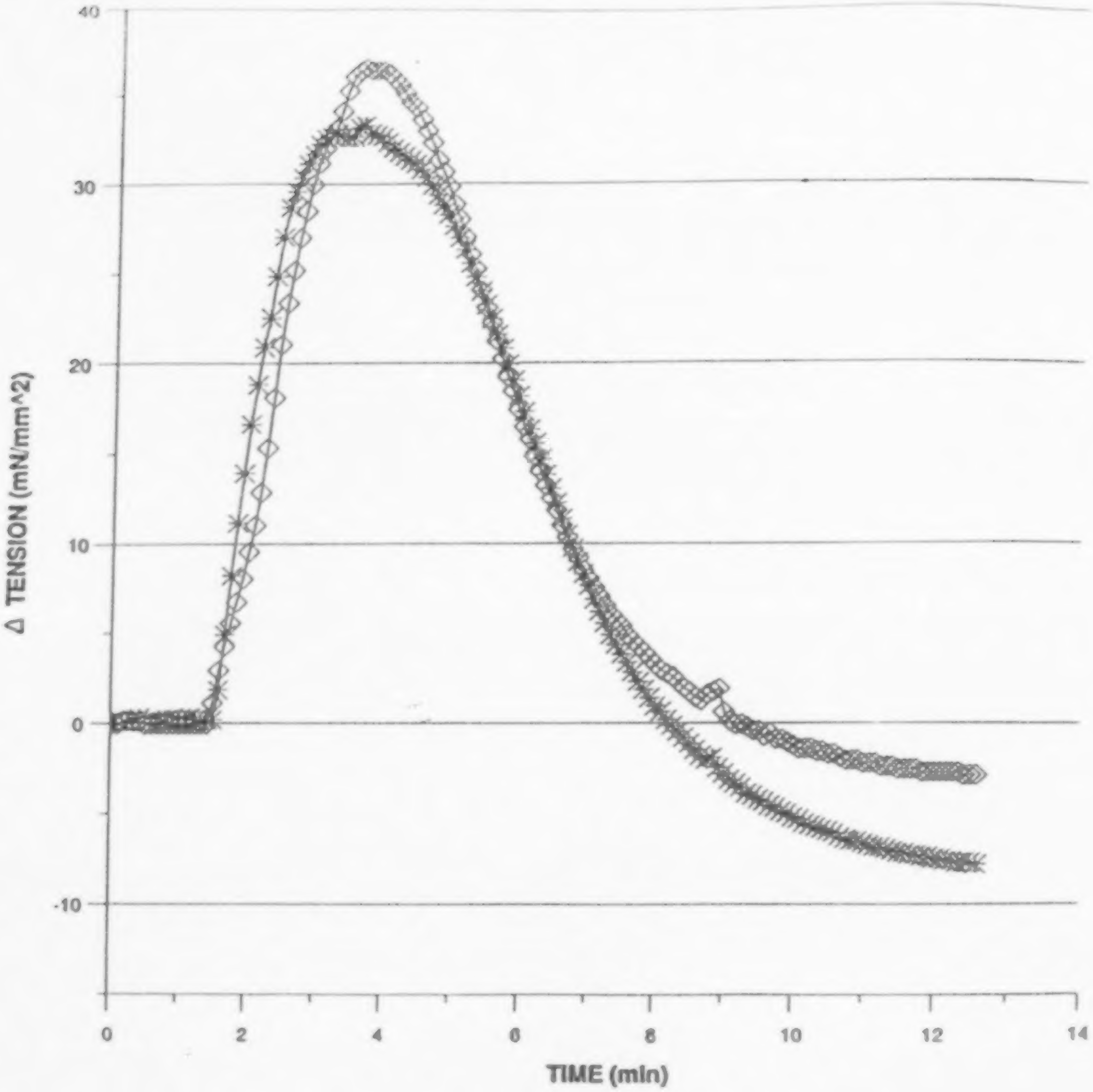

Figure #2.3 Effect of LTD_4 on Isometric Tension in Bovine Trachealis Muscle.

LTD_4 (0.1μM) added at the 1.5 minute mark caused transient isometric tension curves in two (✱ , ◇) individual trachealis muscle strips.

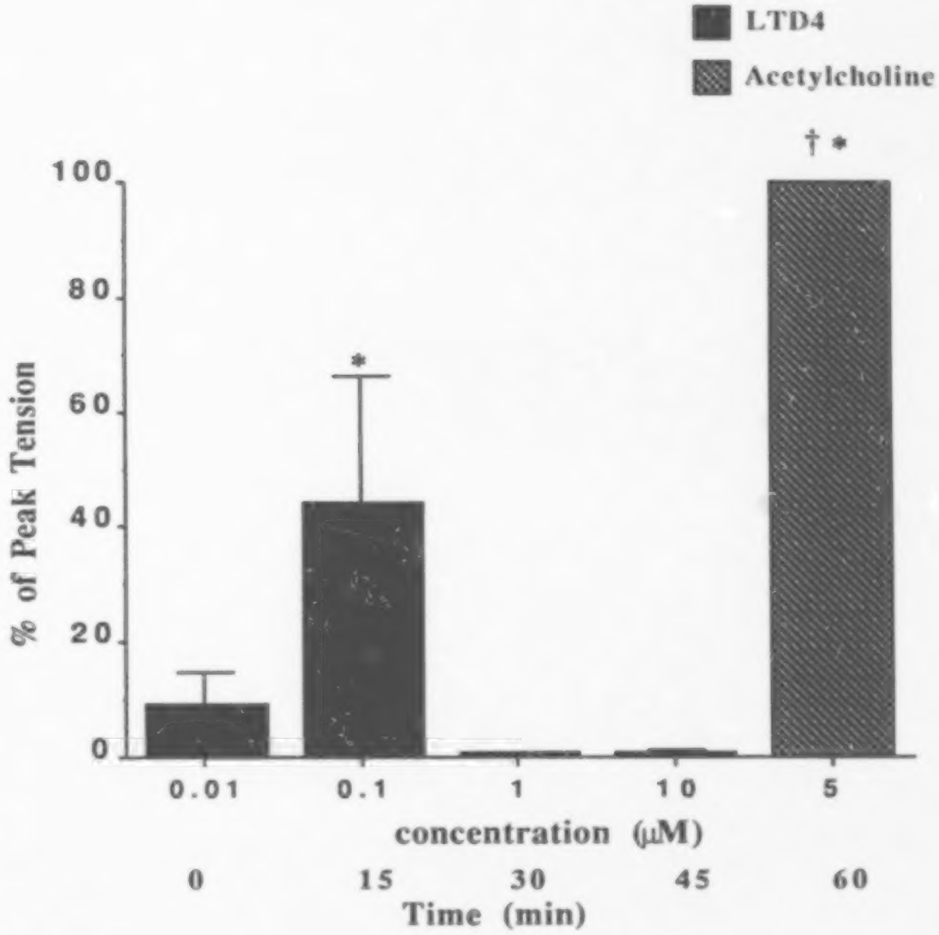

Figure # 2.4 Tension Responses to LTD_4 on Tracheal Smooth Muscle.

Cumulative effects of LTD_4 and ACh on peak tension in the same 4 muscle strips. There is evidence of tachyphylaxis. Also, the peak contraction produced by LTD_4 is much lower than that by ACh. Before ACh stimulation LTD_4 was washed off with two rinses of KH solution. * indicates significance from 0.1 LTD_4 and 5 μM ACh to 0.01, 1, and 10 μM LTD_4., † indicates significance from 0.1 μM LTD_4 to 5 μM ACh (by Newman-Keuls multiple comparison test, critical value of 0.05).

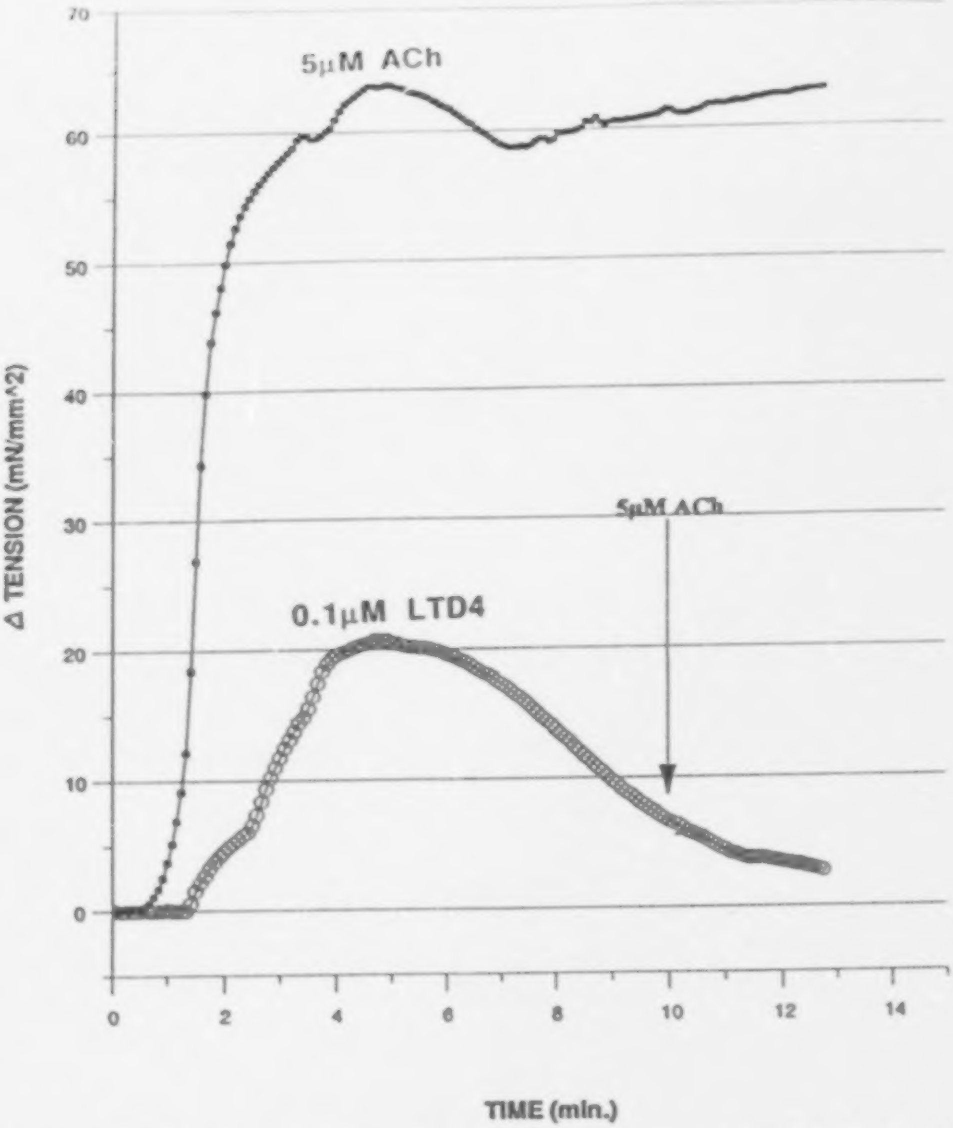

Figure #2.5 Effect of ACh and LTD_4 on Isometric Tension in Bovine Trachealis Muscle.

When ACh (5 μM) was added 9 minutes after LTD_4 (0.1 μM) (-⊖- symbol), which was used to cause a contraction, it produced no contractile response. Using the same tissue (-●- symbol) after 2 rinses with KH solution, the contractile response to ACh was restored.

Effect of PAF on Isometric Tension

Figure 2.6 is a typical isometric tension-time curve caused by PAF (50 μM) stimulation without the addition of platelets to the bathing medium. The tension increased to a peak and was not sustained. In figure 2.7, the first dose of PAF (50 μM) induced a peak tension that was not significantly different from the histamine (3.5 μM)-induced contractions, but the second identical dose of PAF was significantly lower than the first peak response and the two peak responses to histamine (n=6) ($P \leq 0.05$).

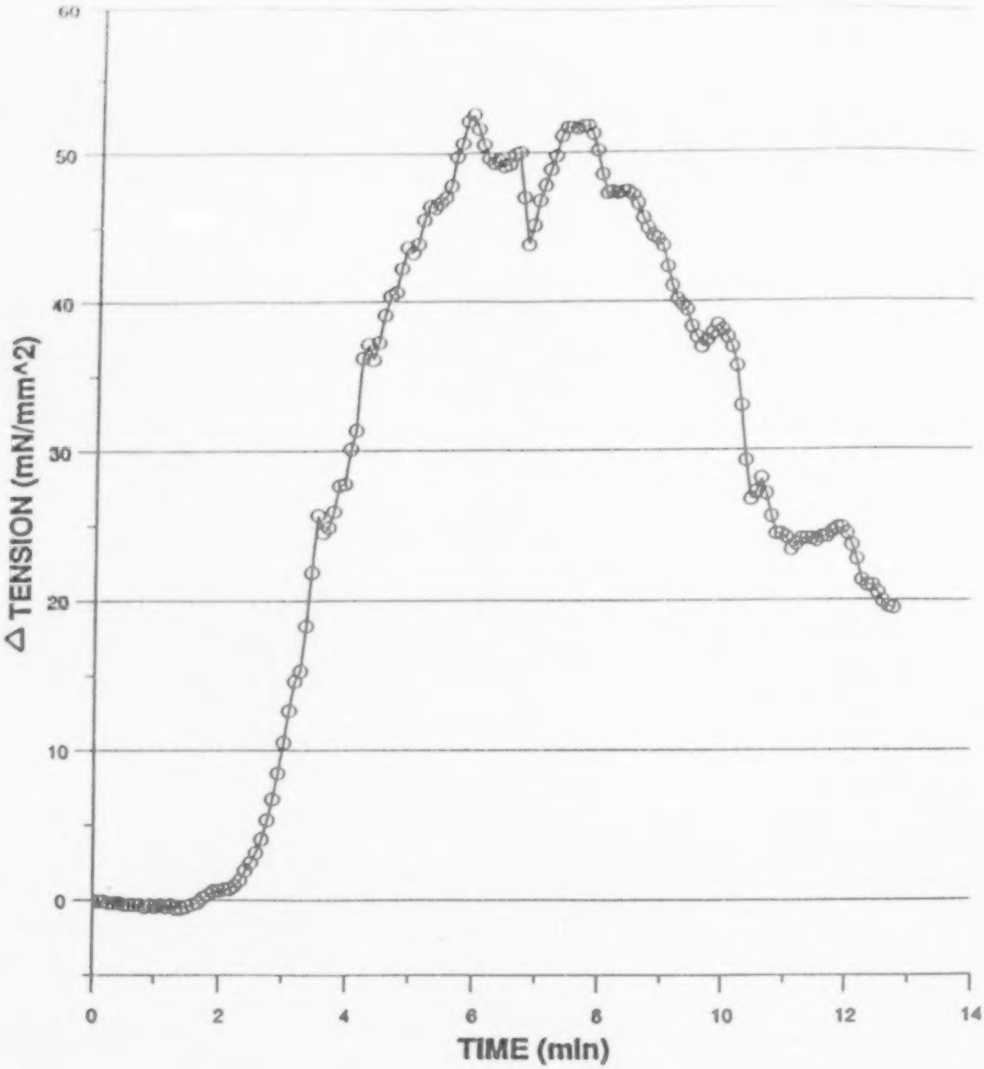

Figure #2.6 Effect of PAF on Isometric Tension in Bovine Trachealis Muscle.

PAF (50 μM) added at the 1 minute mark induced an isometric tension curve. -ϴ- symbol is one trachealis muscle strip. (no platelets added to the bathing medium)

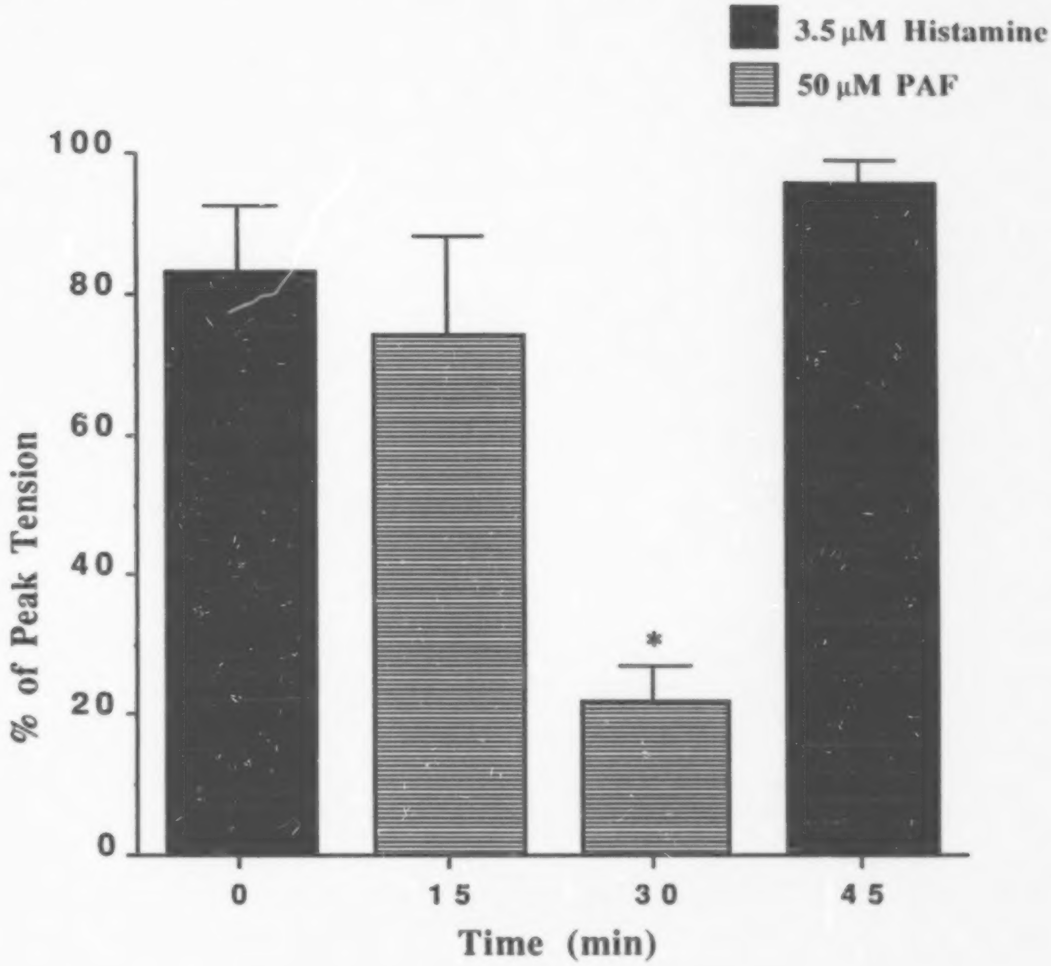

Figure # 2.7 Effect of PAF and Histamine Stimulation on Tracheal Smooth Muscle.

The effect of 50 µM PAF and 3.5 µM histamine on the peak tension was studied in 6 muscle strips (two rinses in KH solution between stimulations). * indicates significance from both 3.5 µM histamine stimulation and the first stimulation of 50 µM PAF (by Newman-Keuls multiple comparison test, critical value of 0.05). Note, platelets were not added to the bathing medium.

Effect of $PGF_{2\alpha}$ on Isometric Tension

Figure 2.8 is typical isometric tension-time curves caused by $PGF_{2\alpha}$ (10 μM) stimulation. The tensions were maintained and peak responses were variable. Figure 2.9 shows the peak tensions induced by cumulative dose of $PGF_{2\alpha}$ (0.01, 0.1, 1, 10 and 100 μM). Both 10 μM and 100 μM of $PGF_{2\alpha}$ caused significantly larger peak tensions compared to the tensions obtained by $PGF_{2\alpha}$ at lower doses (0.01, 0.1 and 1 μM) ($P < 0.05$). In figure 2.10, $PGF_{2\alpha}$ (50 μM)-induced peak tensions were not significantly different from each other, and were not significantly lower than those induced by histamine (3.5 μM) (n=6).

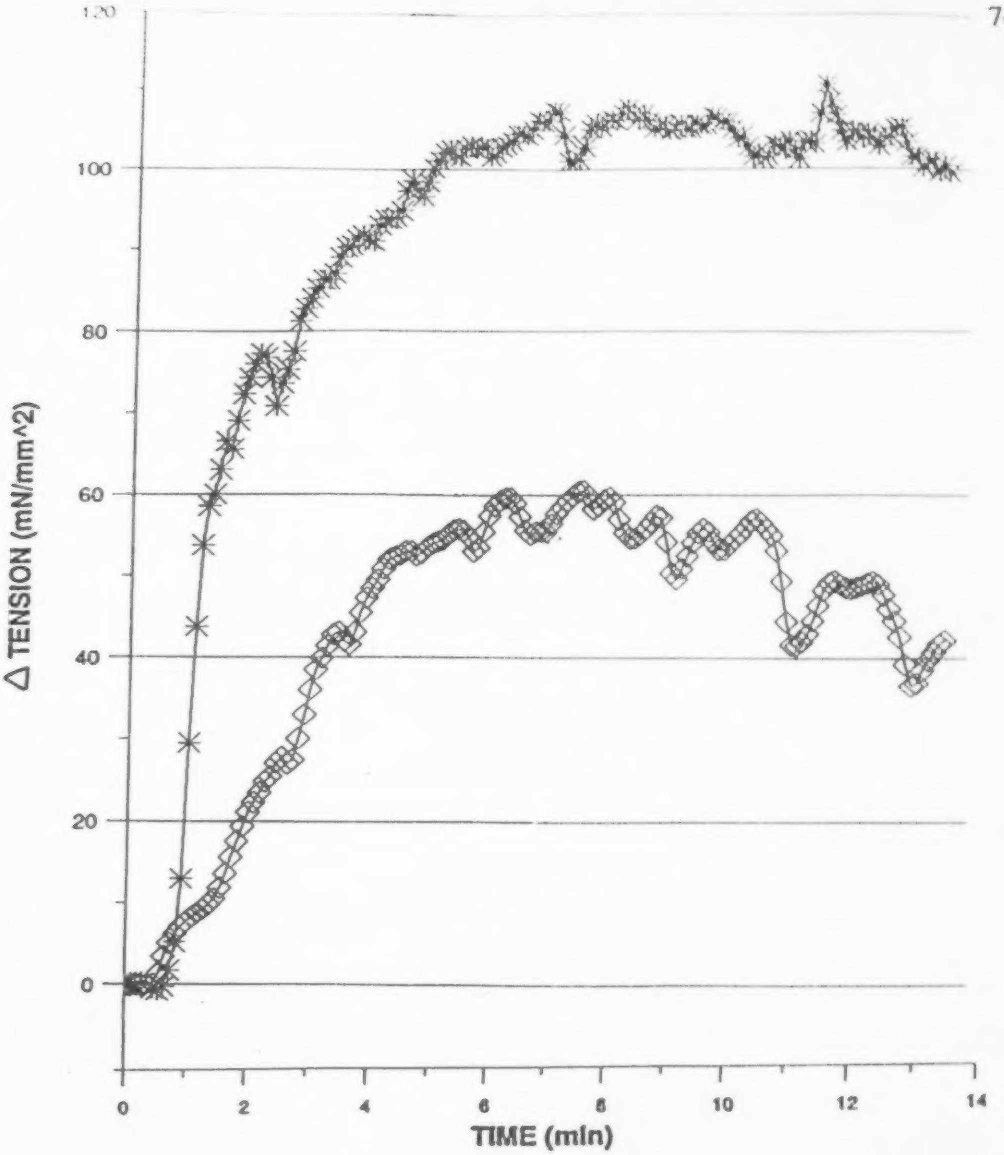

Figure #2.8 Effect of PGF2α on Isometric Tension in Bovine Trachealis Muscle.

PGF2α (10μM) added at the 30 second mark induced isometric tension curves. (✳, ◇) symbols represent individual tensions in two trachealis muscle strips.

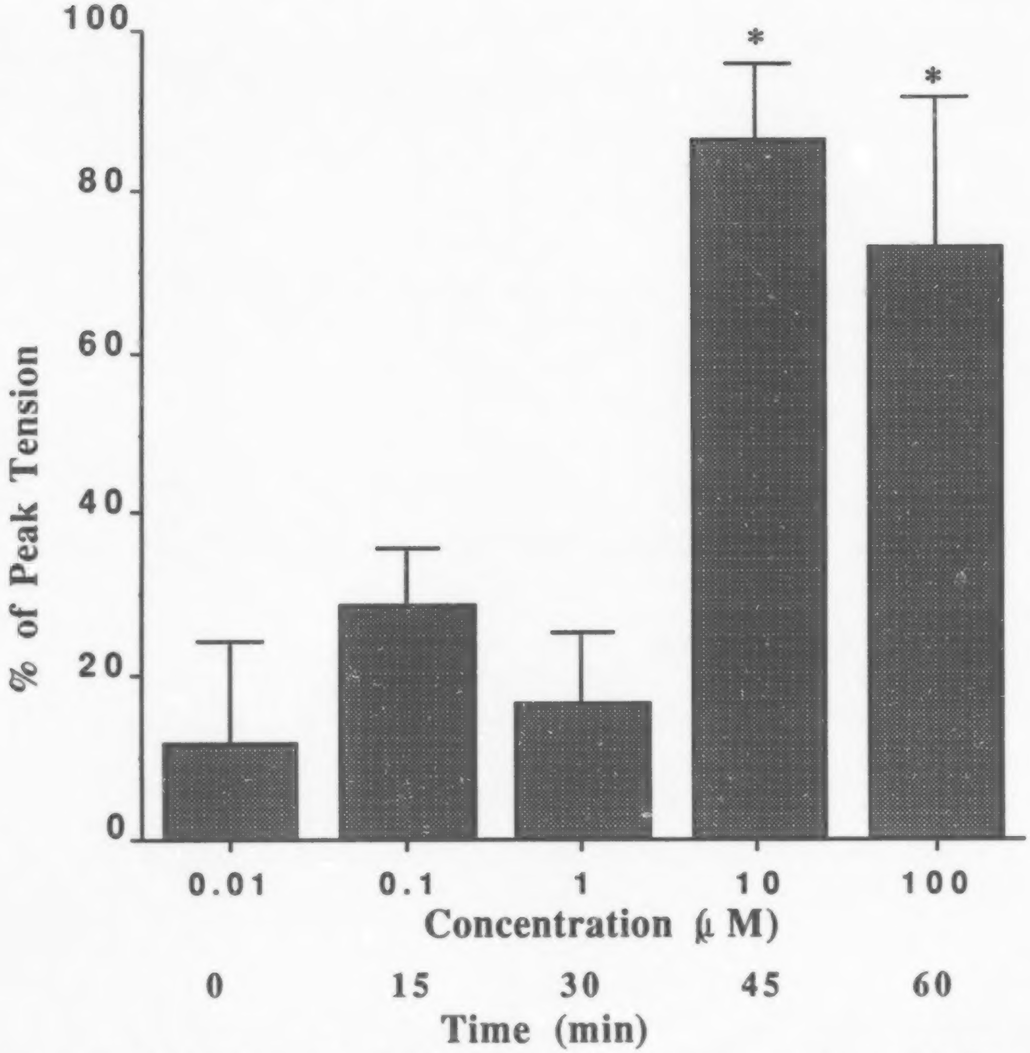

Figure # 2.9 Dose-Responses of Peak Tensions to Increasing Doses of PGF$_{2\alpha}$ on Tracheal Smooth Muscle.

The effect of increasing concentrations (cumulative addition) of PGF$_{2\alpha}$ on the peak tensions in 4 muscle strips. * indicates significant differences from 0.01, 0.1, and 1 μM to 10 and 100 μM PGF$_{2\alpha}$ (by Newman-Keuls multiple comparison test, critical value of 0.05).

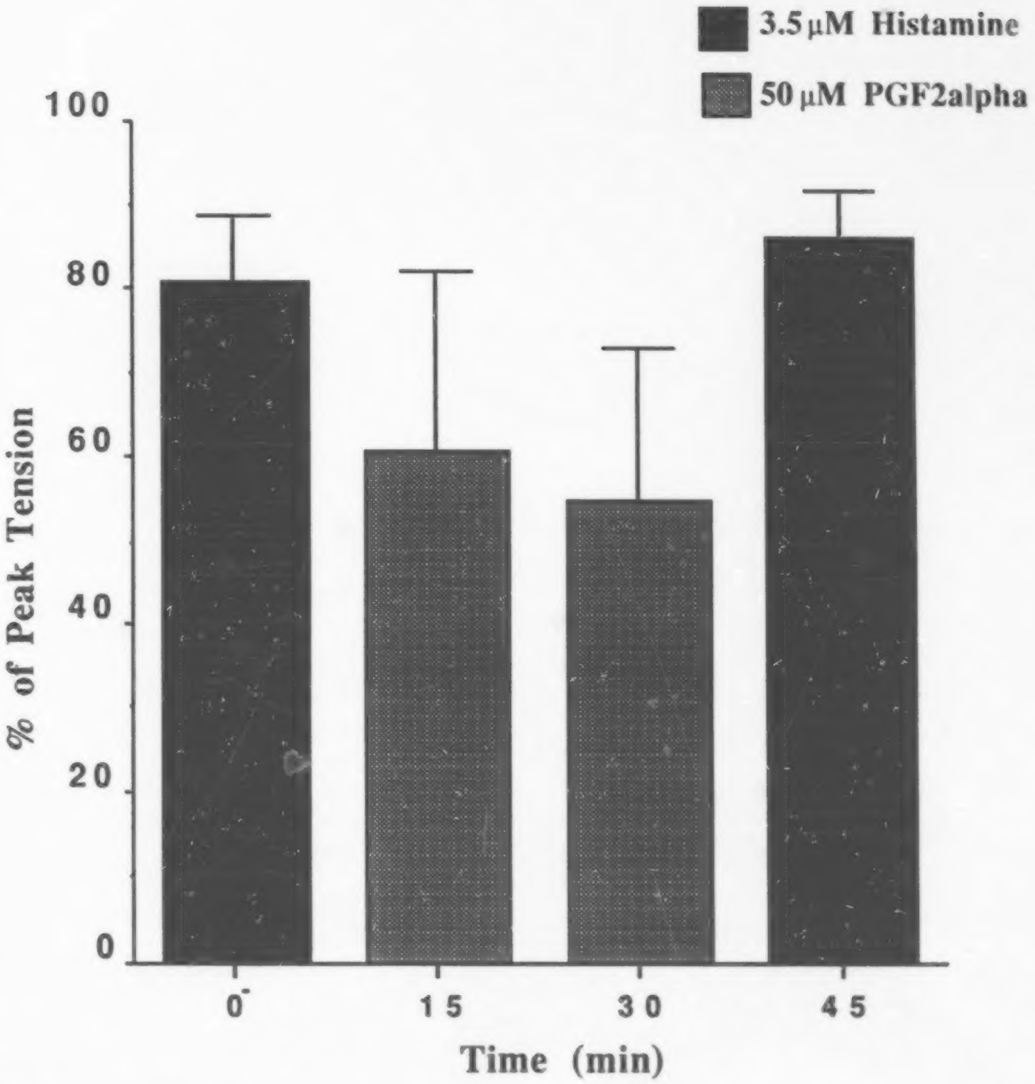

Figure # 2.10 Effect of $PGF_{2\alpha}$ and Histamine Stimulation on Tracheal Smooth Muscle.

The effect of 50 µM $PGF_{2\alpha}$ and 3.5 µM histamine on the peak tension was studied in 6 muscle strips (two rinses of KH solution between stimulations).

Effect of Histamine on Isometric Tension

Figure 2.11 shows a typical tension-time curve response of one muscle strip to histamine (3.5 μM). The tension increased to a peak within 5 minutes of exposure and the tension was sustained. Figure 2.12 shows a dose-response to increasing doses of histamine from (0.001 to 100 μM). The ED50 was estimated from the graph to be approximately 3.5 ± 0.8 μM.

Effect of Repeated Exposures to Histamine

In figure 2.13 a repeated dose of histamine (3.5 μM) on two muscle strips showed no indication of tachyphylaxis.

Effect of Low Calcium on Histamine-Induced Isometric Contraction

Figure 2.14 shows the role of extracellular calcium on the isometric tension-time curve generated by 3.5 μM histamine. When the tissue was bathed in KH solution and contracted with histamine a peak tension and plateau were obtained. However, the tissue when exposed to 3.5 μM histamine in MKH solution for 2 minutes, the tension-time curve showed a decrease rate, a lower peak tension, and no sustained tension.

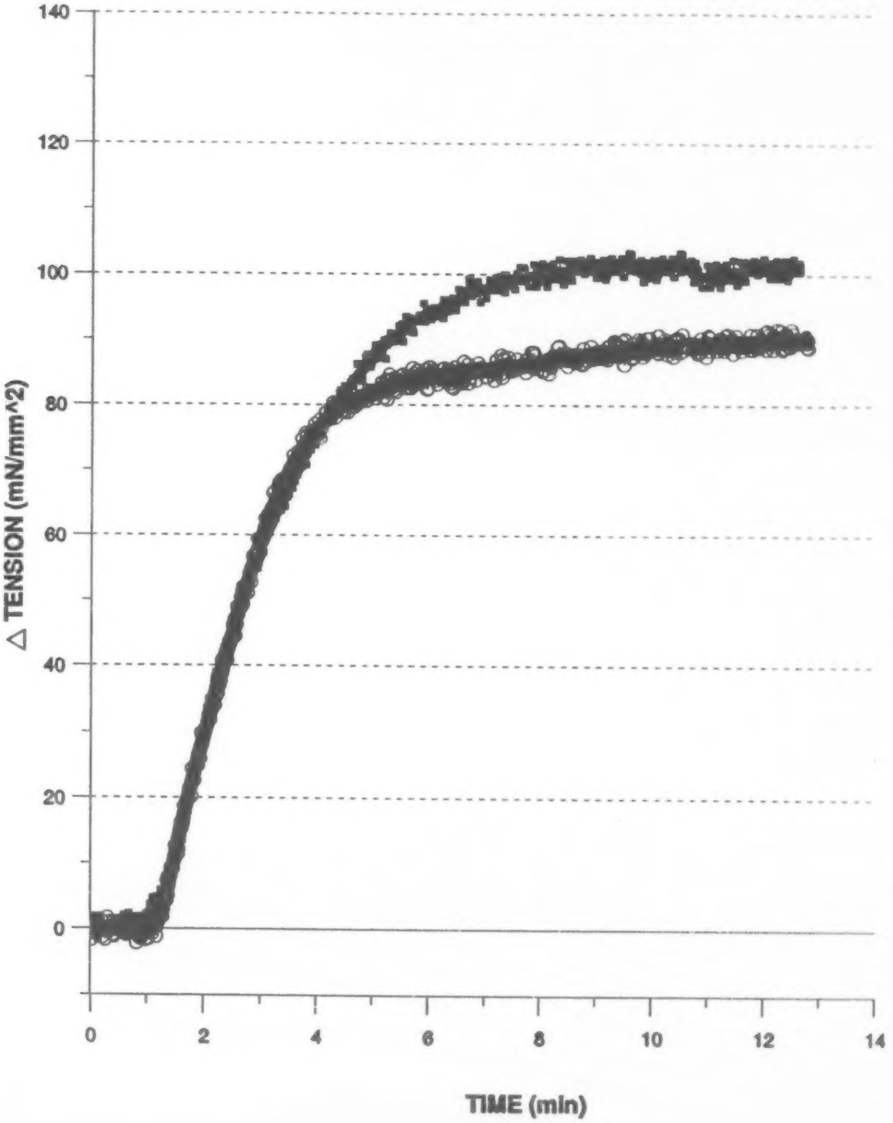

Figure #2.11 Effect of Histamine on Isometric Tension in Bovine Trachealis Muscle.

Histamine (3.5 μM)-induced isometric tension curves from two trachealis muscle strips (tissue 1-○-, tissue 2-■-).

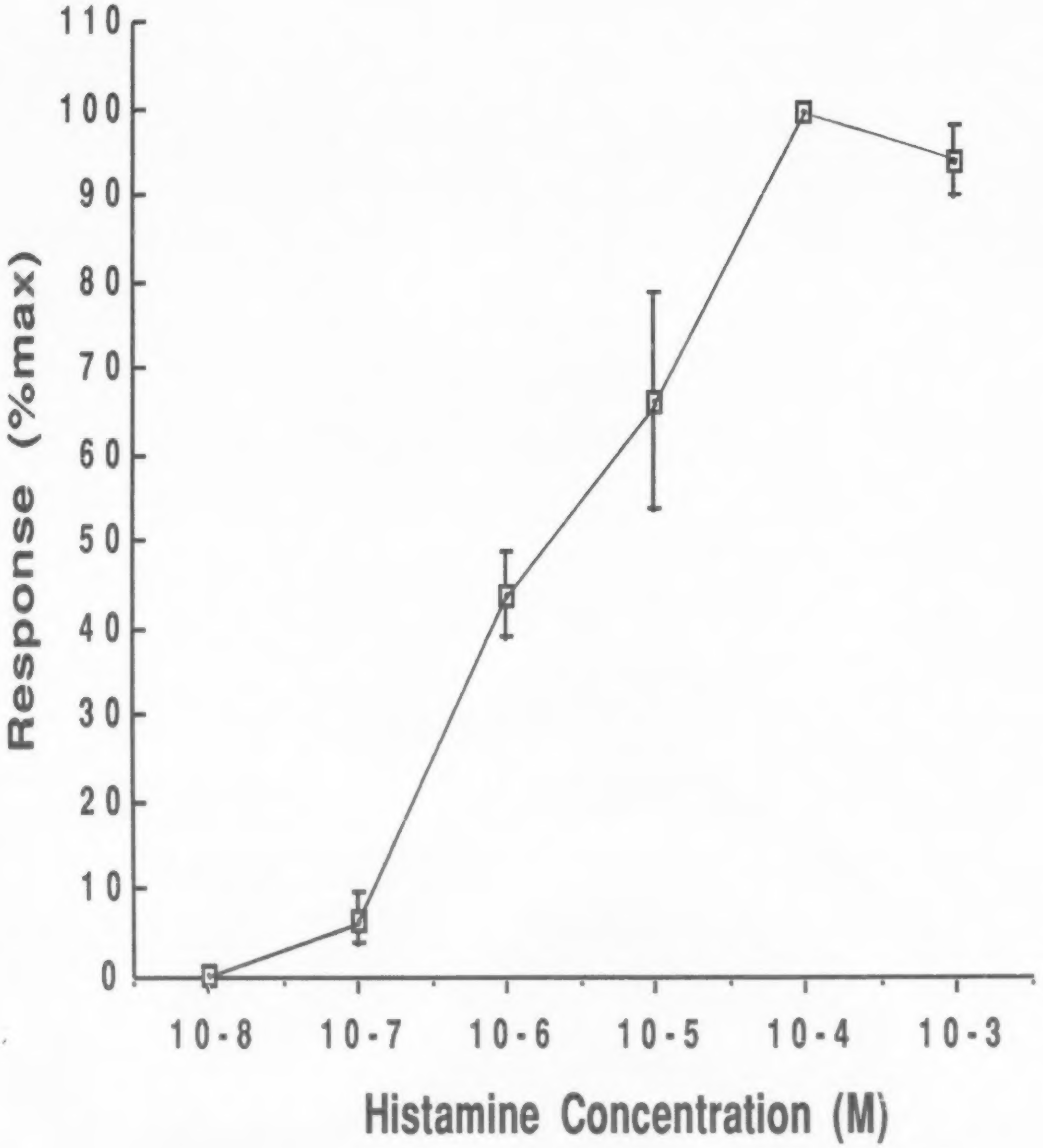

Figure # 2.12 Dose-Responses of Peak Tensions to Increasing Doses of Histamine on Tracheal Smooth Muscle.

The effect of increasing concentrations (cumulative addition) of histamine on the average peak tensions as a percent of maximum (⊟) in 4 muscle strips.

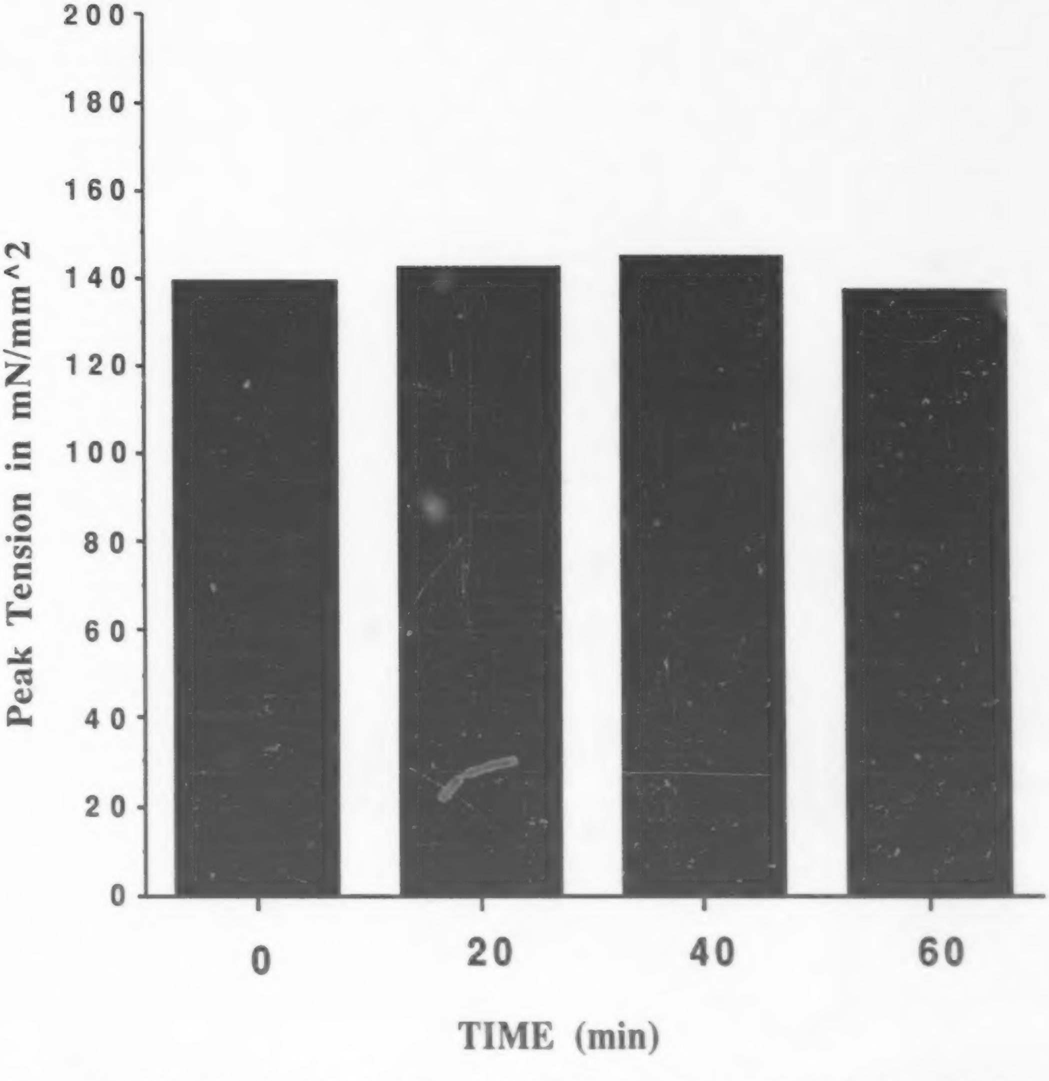

Figure # 2.13 Effect of Histamine on the Average Peak Isometric Tension in Bovine Tracheal Smooth Muscle.

Histamine 3.5 μM was administered at 4 different times to the same two tissue strips. Prior to histamine exposure the muscle strips were rinsed twice in KH solution. Peak tensions are average of the two strips.

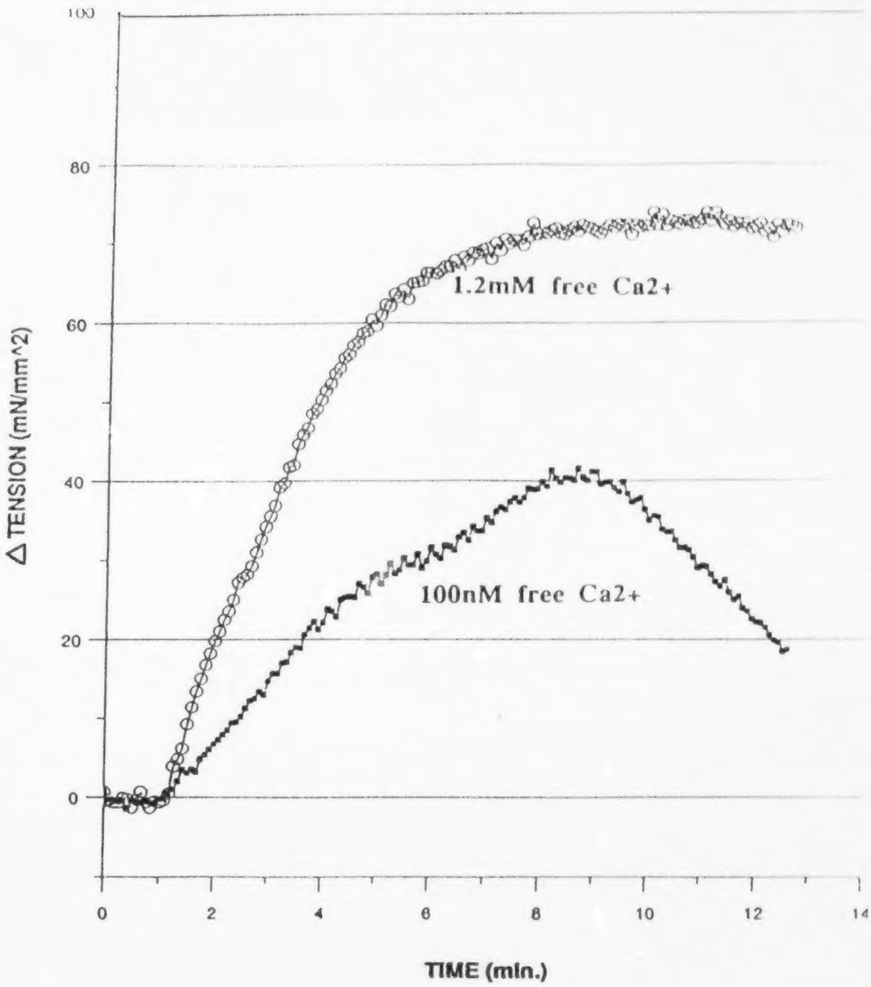

Figure #2.14 Effect of Low Extracellular Calcium on Histamine-Induced Isometric Tension in Bovine Trachealis Muscle.

Exposure to 100 nM free calcium KH solution. Histamine 3.5 μM was administered 2 minutes after low calcium exposure (-●-). The upper tracing is the same piece of tissue previously contracted with 3.5μM histamine in normal KH solution.

Discussion

In the current study on bovine tracheal smooth muscle, I have demonstrated that 1) LTD_4 caused variable isometric contractions and indicated tachyphylaxis. Furthermore, when a tissue has undergone a LTD_4-induced contraction, it could not develop a tension response to ACh, and the response to ACh in the muscle strip was restored when the tissue was rinsed. 2) PAF caused variable isometric contractions without the addition of platelets and indicated tachyphylaxis, 3) $PGF_{2\alpha}$ caused variable isometric contractions and sustained the tension unlike LTD_4 and PAF. 4) Histamine showed a dose-dependant response with no tachyphylaxis, and its contractile properties were largely dependant on the extracellular calcium concentration.

The LTD_4 contractile responses in bovine tracheal smooth muscle suggest that the receptor location is not solely in the parenchymal tissue, and that the parenchymal tissue is not necessary to cause an LTD_4-induced contraction. These results were supported by the work from Mong *et al.*, (1988), which showed that there were [^{3}H] LTD_4 binding stereoselectivity on sheep tracheal smooth muscle cells and that these cells would undergo transient increases in $[Ca^{++}]_i$ when stimulated with LTD_4. These results and the results reported by Doucet *et al.*, (1990) suggest that there could be some species variation in the LTD_4 receptor location.

One other observation regarding the LTD_4 contractile response was the decrease in tension below baseline values after the LTD_4-induced peak response. Also, the LTD_4-induced contraction showed no change in tension when acetylcholine (ACh) was administered after peak tension via LTD_4, but once the tissue was rinsed with Krebs-Henseleit (KH) solution, the same dose of ACh caused a brisk and maintained tension. Because this has not been reported the mechanism in which the inhibition or the LTD_4-induced relaxation operates could be caused by either 1) the formation of a relaxant factor(s) such as prostaglandin E_2 (Madison *et al.*, 1989) or vasoactive intestinal polypeptide (Said, 1988) or an increase in intracellular cyclic AMP concentration, 2) a decrease in calcium sensitivity of the contractile elements (Karaki, 1989) or 3) some unknown mechanisms.

PAF (50 μM) caused a transient increase in isometric tension without the addition of platelets to the bathing medium. After rinsing, the second repeated dose, caused a reduced isometric contraction, which indicates tachyphylaxis. The results obtained using PAF on bovine tracheal smooth muscle suggests that either there were PAF specific receptors on tracheal smooth muscle cell membranes, or that there were some platelets in the tissue, remaining after rinsing, which become activated (release of 5-HT) when PAF was administered. It should be noted that the concentration needed to induce any noticeable contraction by PAF was considerably higher than the concentrations used by Popovich *et al.*, (1988). This could be due to species variation (canine vs. bovine) or could be a function of possibly the small number of platelets in the tissue. To help identify the role of 5-HT in PAF-induced tracheal muscle contraction, it would require a specific 5-HT antagonist for this tissue. This antagonist to date is not available; therefore, the function of 5-HT in PAF-induced contraction could not be properly investigated.

The isometric tension-time curve elicited by $PGF_{2\alpha}$ showed a sustained tension that was not dose-dependant and it caused variable peak tensions. The single dose response (50 showed similar peak responses and suggests that $PGF_{2\alpha}$ is not tachyphylactic and therefore its application does not induce the production of PGE_2 (Douglas and Brink, 1987). The differences shown by my preparation and Douglas and Brink, (1987) may be due to species variations (bovine vs. guinea pig), or to other unknown mechanisms.

Histamine caused a dose-dependant response, were the ED50 was approximated at 3.5 ± 0.8 μM. The histamine-induced contractions showed a sustained tension and did not indicate tachyphylaxis. The response of histamine-induced contraction in low extracellular calcium medium showed a response similarly to Kirkpatrick, (1978) which had a decreased peak response and therefore agrees with the hypothesis that extracellular calcium concentration plays an important role in histamine-induced isometric contraction in bovine tracheal smooth muscle.

In summary, I found using bovine tracheal smooth muscle an increase in isometric tension when it was stimulated by LTD_4, PAF (without the addition of platelets to the bathing medium), $PGF_{2\alpha}$ (which showed a maintained tension unlike LTD_4 and PAF) and histamine (which caused a dose-dependant response and no signs of tachyphylaxis.) In the doses used, LTD_4, PAF and $PGF_{2\alpha}$ the muscle produced a smaller tension than that achievable with histamine or acetylcholine.

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Chapter III

Fluo-3 as an Indicator for Simultaneous Measurement of $[Ca^{++}]_i$ and Isometric Tension in Histamine Stimulated Bovine Tracheal Smooth Muscle

Introduction

Measurements of intracellular calcium concentration ($[Ca^{++}]_i$) have advanced our understanding of how calcium modulates the excitation-contraction coupling / uncoupling mechanism in airway smooth muscle (Taylor and Stull, 1988; Lofdahl and Barnes, 1986; Panettieri *et al.*, 1989). Recently bioluminescent and fluorescent techniques became available for the simultaneous measurement of $[Ca^{++}]_i$ and isometric tension in airway smooth muscle (Tukawa *et al.*, 1987; Gunst, 1989; Ozaki *et al.*, 1990). Their findings have shown that the role of $[Ca^{++}]_i$ in the excitation-contraction coupling and uncoupling mechanism in airway smooth muscle is multidimensional, and worthy of further investigation.

Fluo-3 as an $[Ca^{++}]_i$ Indicator

To measure $[Ca^{++}]_i$ in bovine tracheal smooth muscle contraction induced by histamine a relatively new fluorescent indicator fluo-3 (Minta *et al.*, 1989) was used. Fluo-3 has been used to monitor $[Ca^{++}]_i$ in fibroblasts, T-lymphocytes (Kao *et al.*, 1989), platelets and neutrophils (Merritt *et al.* 1990), and in colonic smooth muscle (Gerthoffer *et al.*, 1991) but it has not been used in airway smooth muscle tissue preparations.

There are three main advantages of using fluo-3 to measure $[Ca^{++}]_i$: 1) the excitation wavelength of fluo-3 is outside the UV range; therefore, NADH will not offset the measurement as it would with the fluorescent dye fura II (Ozaki *et al.*, 1987; Sato *et al.*, 1988). Also, fluo-3 should not cause cellular damage, which is associated with UV exposure (Minta *et al.*, 1989; Williams and Fay, 1986). 2) fluo-3 allows for a broad range of $[Ca^{++}]_i$ measurement, from ≈ 35 nM to 10 μM (Minta *et al.*, 1989; Kao *et al.*, 1989), which is an advantage in the present study, 3) fluo-3 has a high Kd (dissociation constant) which is important in achieving optimal measurement of the fluorescent maximum in tissue work.

Histamine as a Contractile Agonist

Histamine has been shown to cause airway smooth muscle excitation/contraction coupling *in vitro* (Kirkpatrick, 1975; Katsuyama *et al.*, 1990) via its action on the H_1 receptor, (Ash and Schild, 1966; Chand and Eyre, 1975) which has been shown to cause an increase in the concentration of the second messenger, inositol 1,4,5 trisphosphate (IP_3) (Barnes *et al.*, 1986; Hall and Hill, 1988). IP_3 then causes an increase in $[Ca^{++}]_i$ resulting in muscle contraction. It is postulated that this IP3-initiated increase in $[Ca^{++}]_i$ originates from myoplasmic stores (sarcoplasmic reticulum) (Takuwa *et al.*, 1987; Kotlikoff *et al.*, 1987). Also, the influence of extracellular calcium on bovine tracheal smooth muscle contraction, caused by histamine, was demonstrated by extracellular calcium depletion and calcium channel blocker experiments (Kirkpatrick, 1975). Kirkpatrick, 1975 showed that the height and maintenance of tension were associated to extracellular calcium.

In this study, using bovine tracheal smooth muscle, my **aims** were: 1) to determine if fluo-3 (in the AM ester form) has any inherent effects on histamine-induced contraction, 2) to determine if fluo-3 is suitable in detecting changes in

$[Ca^{++}]_i$ caused by histamine-induced contraction, and 3) to monitor the continuous simultaneous measurements of $[Ca^{++}]_i$ and isometric tension.

Materials and Methods

Fluo-3 acetoxymethyl ester (AM) and fluo-3 pentaammonium salt were purchased from Molecular Probes Inc. (Eugene, OR., USA); pluronic F-127 was supplied by Molecular Probes. Histamine phosphate and $MnCl_2$ were purchased from Sigma (St. Louis, MO., USA). Ionomycin was purchased from Terochem Laboratories (Edmonton, AB. CANADA). Krebs-Henseleit buffer (KH) for these experiments contained (in mM) 113 NaCl, 4.8 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, and 5.5 glucose.

Smooth Muscle Isolation

This method was identical to that described in chapter II.

Simultaneous Measurement of $[Ca^{++}]_i$ and Isometric Tension.

A muscle strip 1 mm wide x 1 mm thick x 10 mm long, wet weight $\simeq$ 7 to 15 mg, was mounted on an attachment unit (CT-01; Jasco INC. Maryland, USA), with one end free for later attachment to an adjustable length force transducer (FT-03; Grass Instrument, Quincy, MA, USA). The attachment unit was then placed in a cuvette and installed in the calcium ion analyzer (CAF-100; Jasco INC. Maryland, USA) (see figure 3.1).

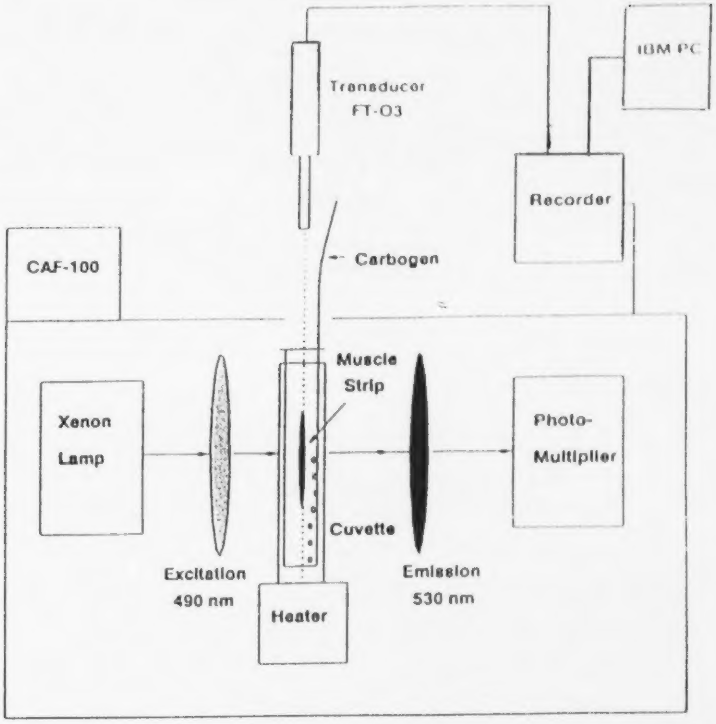
Transducer
FT-03
IBM PC
Recorder
CAF-100
Carbogen
Muscle
Strip
Xenon
Lamp
Photo-
Multiplier
Cuvette
Excitation
490 nm
Emission
530 nm
Heater

Figure # 3.1 CAF-100 Calcium Ion Analyzer with Tension Transducer.

A 75 watt xenon light source was aligned through a rotating excitation filter of 490 nm. The light source aperture was set at 15 mm and the light passed through the cuvette which housed the tissue strip. The muscle strip was centred in the light beam which had a diameter of 4 mm. The fluorescence emitted from the tissue strip was passed through a selective emission filter of 530 nm and detected by the photomultiplier tube. Both tension and fluorescent intensity were monitored on a chart recorder (Gould 3400 RS) and also stored on an IBM computer.

The tissue strip was bathed in 2.5 mls of KH solution and oxygenated with 95% O_2 - 5% CO_2 (carbogen) and kept at pH 7.4. The temperature was maintained at 37 °C. Smooth muscle equilibration was performed identically to the procedure in chapter II. After a stable L_o was established isometric contraction and autofluorescence during histamine (3.5 μM) stimulation was measured. The muscle strip was then rinsed three or four times with KH solution to reestablish the L_o. To load the dye, 15 μM fluo-3 AM in 0.10 % (w/v) pluronic F-127 (a dispersing agent) was added to the KH solution which was kept at room temperature for approximately 3 hours. After this, the dye solution was removed by rinsing the cuvette thoroughly 4 times with KH solution. The cuvette temperature was then raised to 37 °C and 20 minutes later the tissue was stimulated with histamine (3.5 μM). Fluorescent intensity (F) and isometric tension were simultaneously recorded. The apparatus (FT-03 and CAF-100) delay times for both measurements were determined and found to be negligible for F, and ± 2 seconds for isometric tension. Recordings began 1 minute before histamine was added and lasted for 11 minutes thereafter.

Calibration of fluo-3 fluorescence was done on each individual muscle strip at the end of the study using techniques similar to those of Kao *et al.*, (1989) and Merritt *et al.*, (1990). The maximum fluorescence (Fmax) was obtained by adding ionomycin to the cuvette in increasing concentrations ($\mathrm{Fmax}_{(i)}$), and adding the change in F caused by fluorescent decay from baseline to the time at Fmax (X) (see figure 3.7). In 3 experiments further addition of digitonin (90 μM), which has been shown to increase the F to a value higher then $\mathrm{Fmax}_{(i)}$ did not raise the

Fmax(i) (Merritt *et al*., 1990). The Fmax(i) stabilized within 10 to 15 minutes after the addition of ionomycin. The fluorescent minima (Fmin) was assumed to be the autofluorescence of the tissue (Merritt *et al.*, 1990) and was verified using EGTA (4 mM).

The formula used to calculate the $[Ca^{++}]_i$ was: $[Ca^{++}]_i = Kd\,(F\text{-}Fmin/Fmax\text{-}F)$ (Grynkiewicz *et al.* 1985). The dissociation constant (Kd) of Fluo-3 at 37 °C was calculated to be 593 nM, using methods previously described by Grynkiewicz *et al.*, (1985) and using the ratio of change from 22 °C to 37 °C for fluo-3 (Merritt *et al.*, 1990). After completion of Fmax(i), each muscle strip was washed and removed from the apparatus. The cross sectional area (CSA) was then measured.

Statistical Analysis

Data are presented as mean ± SEM. Comparisons of isometric force, with and without fluo-3, in the same tissue were made using the paired *t* test. Comparisons of baseline and peak $[Ca^{++}]_i$ values during histamine-induced contractions (in the same tissue) were also by paired *t* test. Significance was defined as $P \leq 0.05$.

Results

Fluo-3 Properties

The dissociation constant (K_d) of fluo-3 at 22 °C was found to be 275 nM, using a method similar to Grynkiewicz *et al.*, (1985). The study was done at 37 °C, and the K_d, using the ratio of change from 22 °C to 37 °C for fluo-3 (Merritt *et al.*, 1990) was calculated to be 593 nM. The pharmacologic agents used in this study did not cause fluorescence at the excitation wavelength (490 nm) nor did they cause any significant autofluorescence in the trachealis muscle.

Fluo-3 Effect on Histamine-Induced Isometric Tension

The mean of the peak histamine (3.5 μM)-induced isometric tension in five muscle strips, before (control) and after fluo-3 AM loading, were not significantly different (figure 3.2). Figure 3.3 shows the mean of the initial (from approximately 20 seconds to 2 minutes) isometric tension-time curves for the same five muscle strips from figure 3.2. Both control and dye-loaded muscle strips were not significantly different.

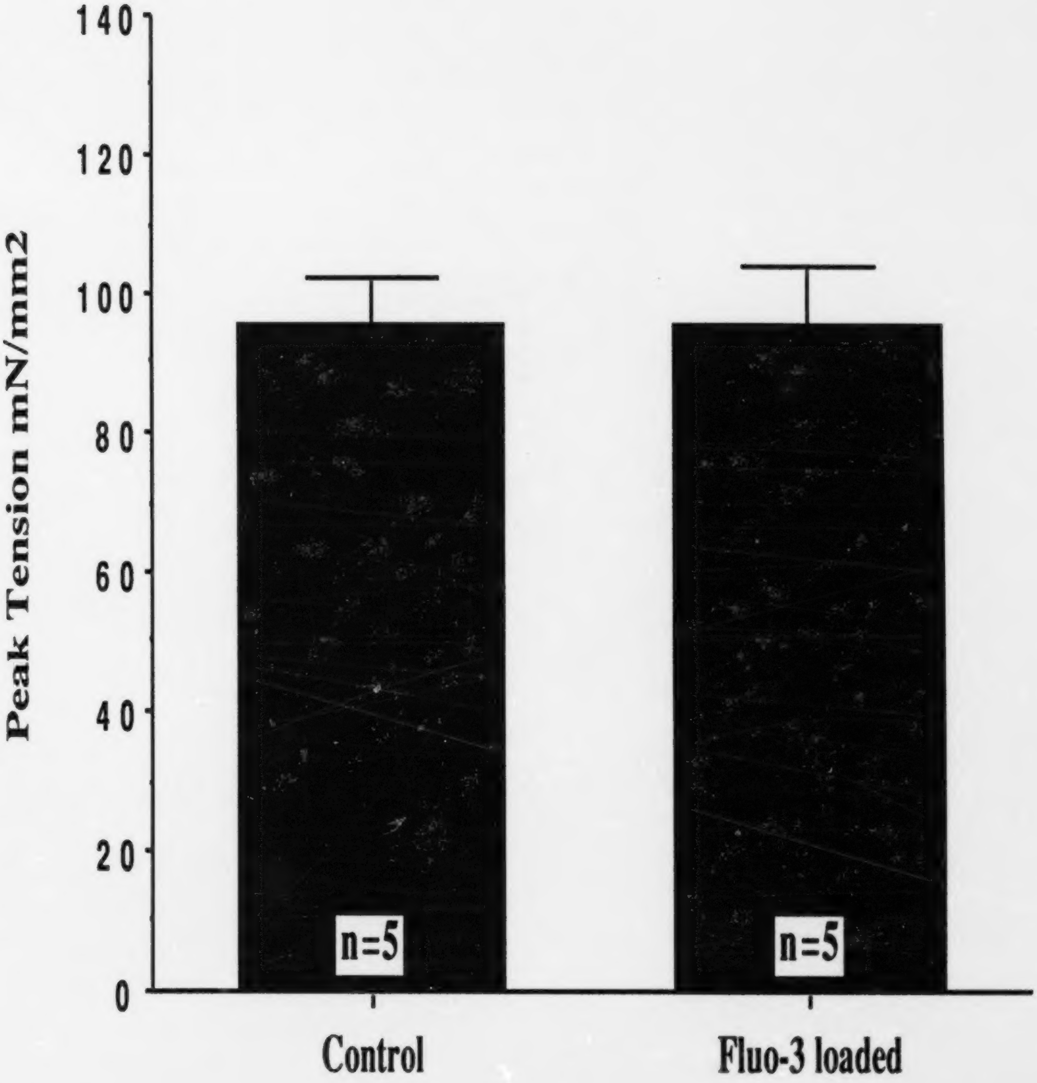

Figure # 3.2 Effect of Fluo-3AM - Pluronic F-127 on Isometric Tension Elicited by Histamine in Bovine Trachealis Muscle.

The peak isometric tension in 5 pieces trachealis muscle contracted with 3.5 μM histamine before (control) and after fluo-3 loading. Values are in mean ± S.E.M.

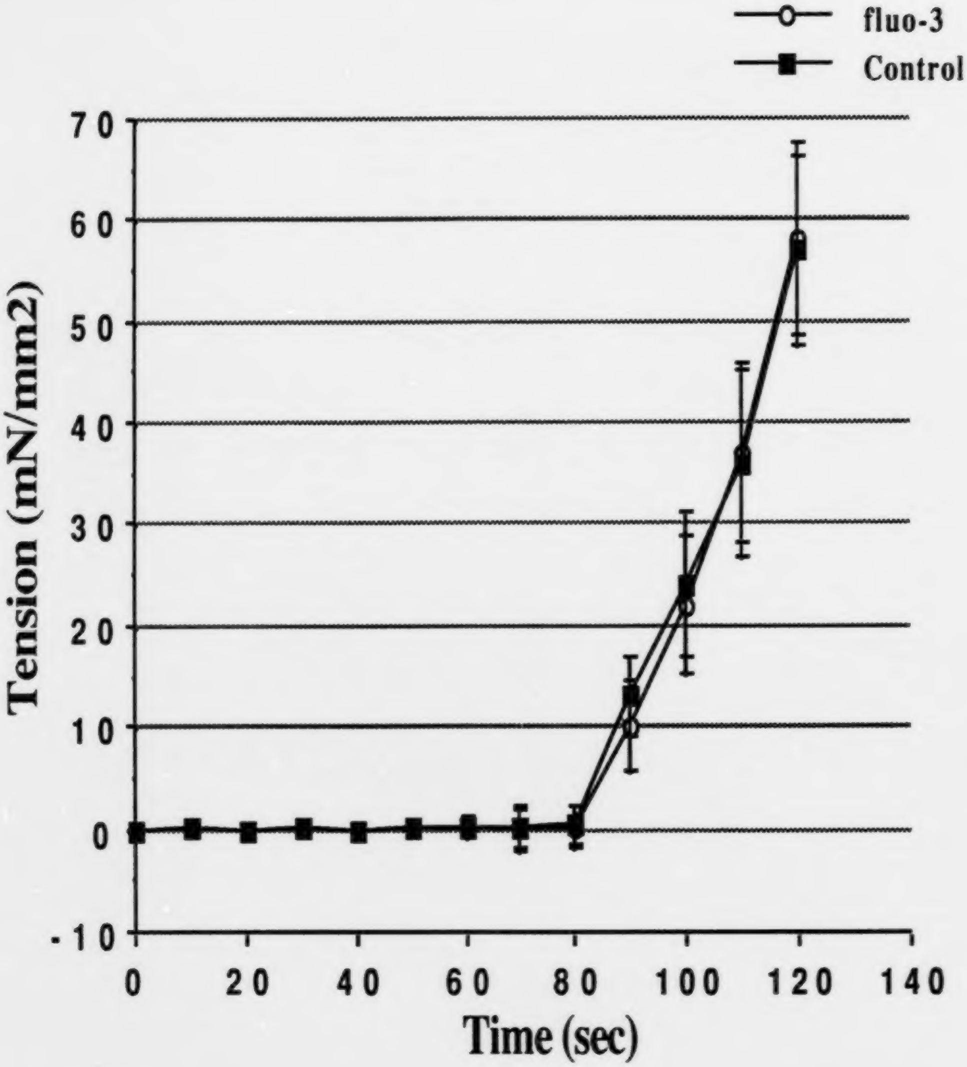

Figure # 3.3 Effect of Histamine on Control and Fluo-3 loaded Bovine Trachealis Muscle.

The isometric tension-time curve from 20 seconds to 2 minutes, of the mean tensions of the same 5 trachealis muscle strips from figure 3.2 (same data). Values are in mean ± S.E.M.

$[Ca^{++}]_i$ Changes Caused by Histamine

Histamine (3.5 µM), added 1 minute after baseline values, caused a significant increase in peak $[Ca^{++}]_i$ for five muscle strips (figure 3.4), $P < 0.05$.

$[Ca^{++}]_i$ and Isometric Tension in Histamine Induced Contraction

Figure 3.5 shows the simultaneous measurements of $[Ca^{++}]_i$ in nM and isometric tension in mN/mm^2 in 5 muscle strips. The baseline $[Ca^{++}]_i$ level declined at a slow rate after fluo-3 was removed from the tissue bath by rinsing thoroughly with KH solution. Following this, addition of histamine (3.5 µM) caused a rapid increase in $[Ca^{++}]_i$ to a peak value which was followed by a slow decline, the rate of which was similar to that seen in the pre-contracted period. Isometric tension was stable before histamine stimulation, but then showed a slow increase after histamine to a plateau that was maintained. Figure 3.6 is the same data as shown in figure 3.5, but the tension and $[Ca^{++}]_i$ values are expressed as a percentage of their maximum values. This better illustrates the time lag of tension increase compared to the increase in $[Ca^{++}]_i$. It shows (figure 3.6) that the $[Ca^{++}]_i$ increased approximately 8 ± 2 seconds prior to the increase in isometric tension.

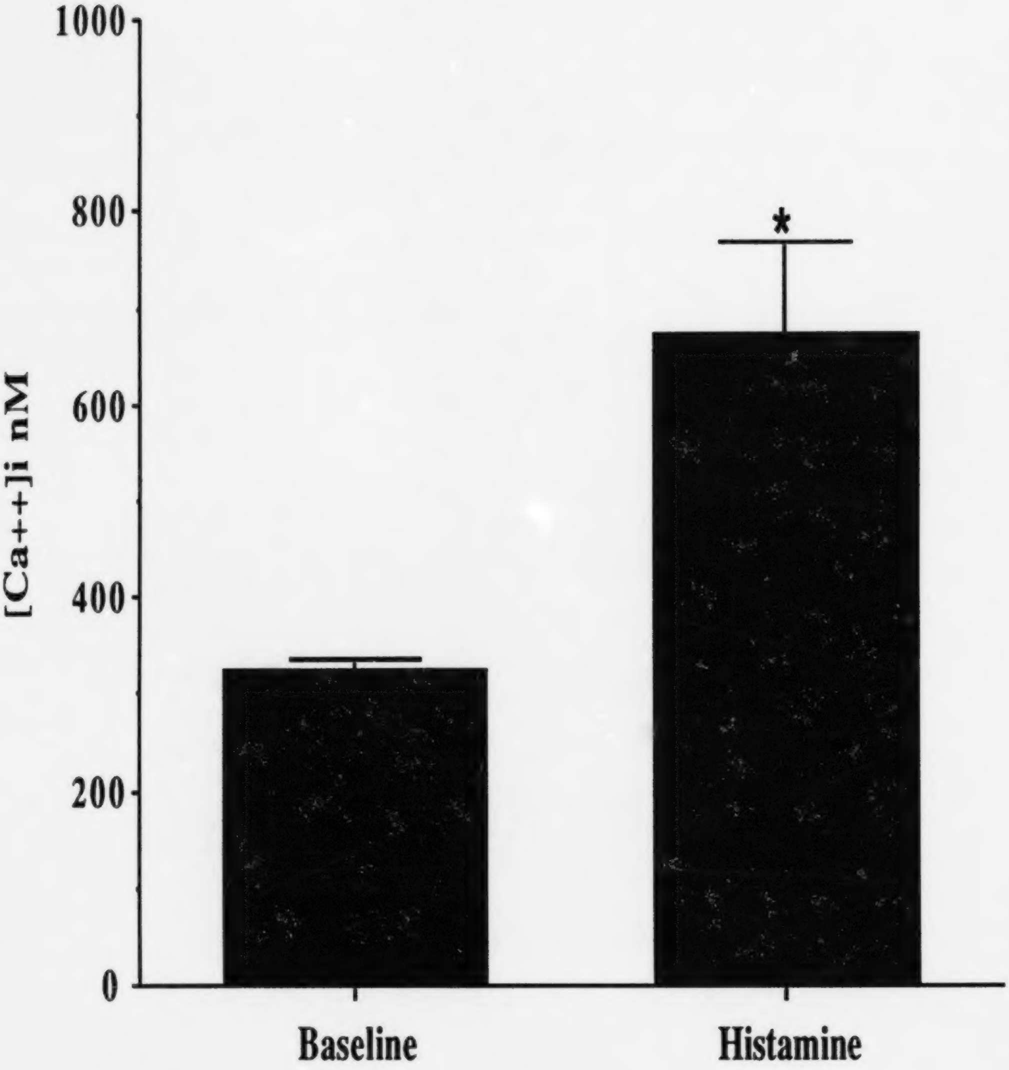

Figure #3.4 Effect of Histamine on Intracellular Calcium in Bovine Trachealis Muscle.

Baseline intracellular calcium level at Lo, and following histamine (3.5 μM) stimulation in 5 trachealis muscle strips. * indicates significance at $p \leq 0.05$.

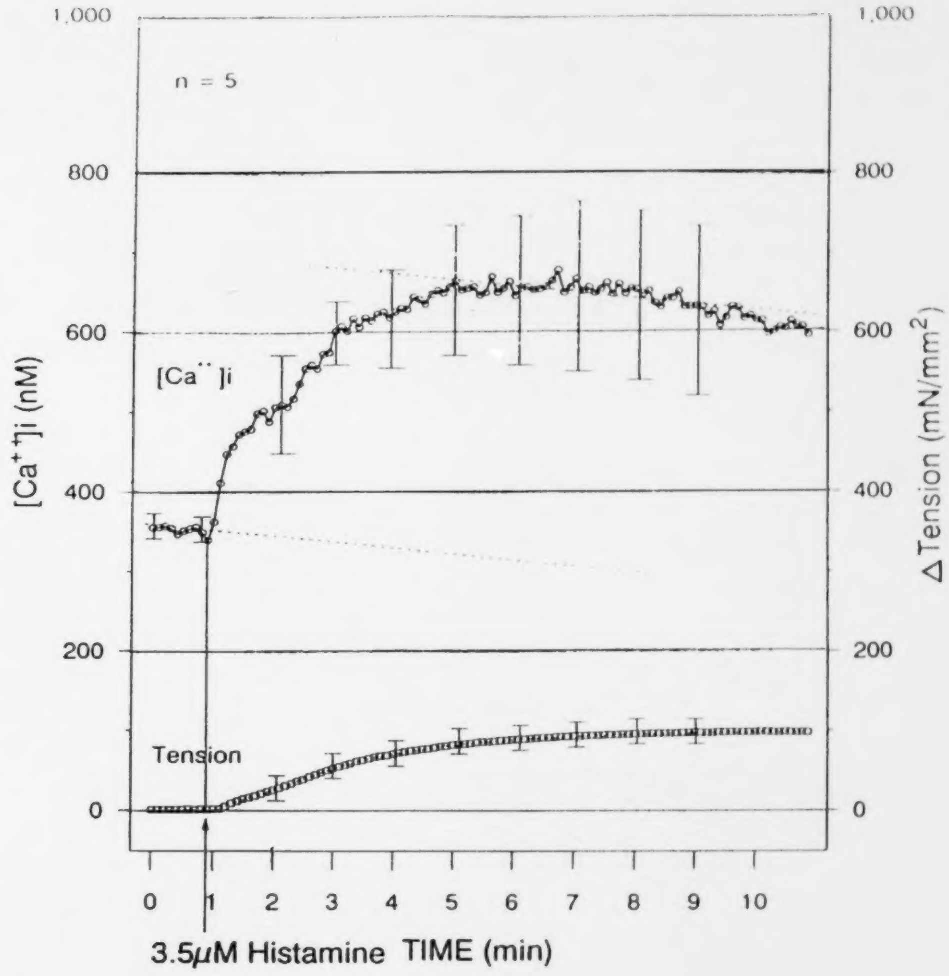

Figure #3.5 Simultaneous Intracellular Calcium and Isometric Tension in Bovine Trachealis Muscle.

Effect of 3.5 μM histamine on [Ca++]i and isometric tension in 5 trachealis muscle strips (--------- is the slope of fluorescent decay). Values are in mean ± S.E.M.

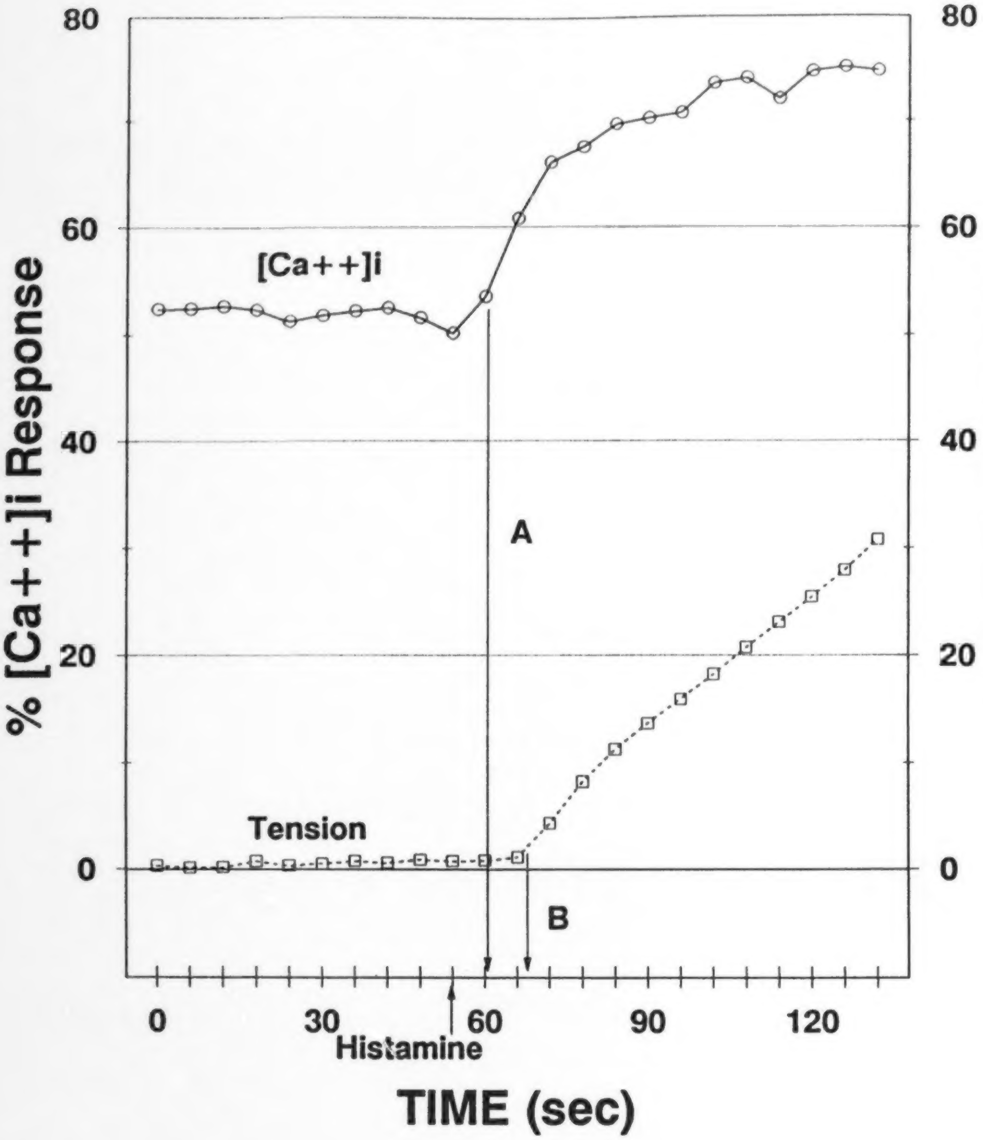

Figure #3.6 Simultaneous [Ca++]i and Isometric Tension Time 0 to 120 seconds in Bovine Trachealis Muscle.

Effect of 3.5 μM histamine on [Ca++]i and isometric tension in 5 trachealis muscle strips, point A is the initial increase of [Ca++]i, point B is the initial increase in isometric tension. Same data points from figure 3.5.

Calibration of Fluo-3 Fluorescence

With the tissue strip in histamine-induced contraction, Fmax was calculated after adding 60 μM ionomycin (figure 3.7). Ionomycin, which is a calcium ionophore, caused a rapid increase in F to a maintained plateau ($Fmax_{(i)}$). Administration of 5 mM $MnCl_2$, to quench the fluo-3, caused a rapid decline in F to just above the autofluorescence of the tissue strip. The actual Fmin was not obtained, because $MnCl_2$ does not quench the dye completely (Kao *et al*., 1989), therefore the autofluorescent value of the tissue strip was used as the fluorescent minima (Fmin) (Merritt *et al.*, 1990). In several muscle strips, addition of 90 μM digitonin did not further increase the fluorescent maximum when added after 60 μM ionomycin, as previously reported by Merritt *et al*., (1990). Evidentially, $Fmax_{(i)}$ could be obtained with ionomycin alone.

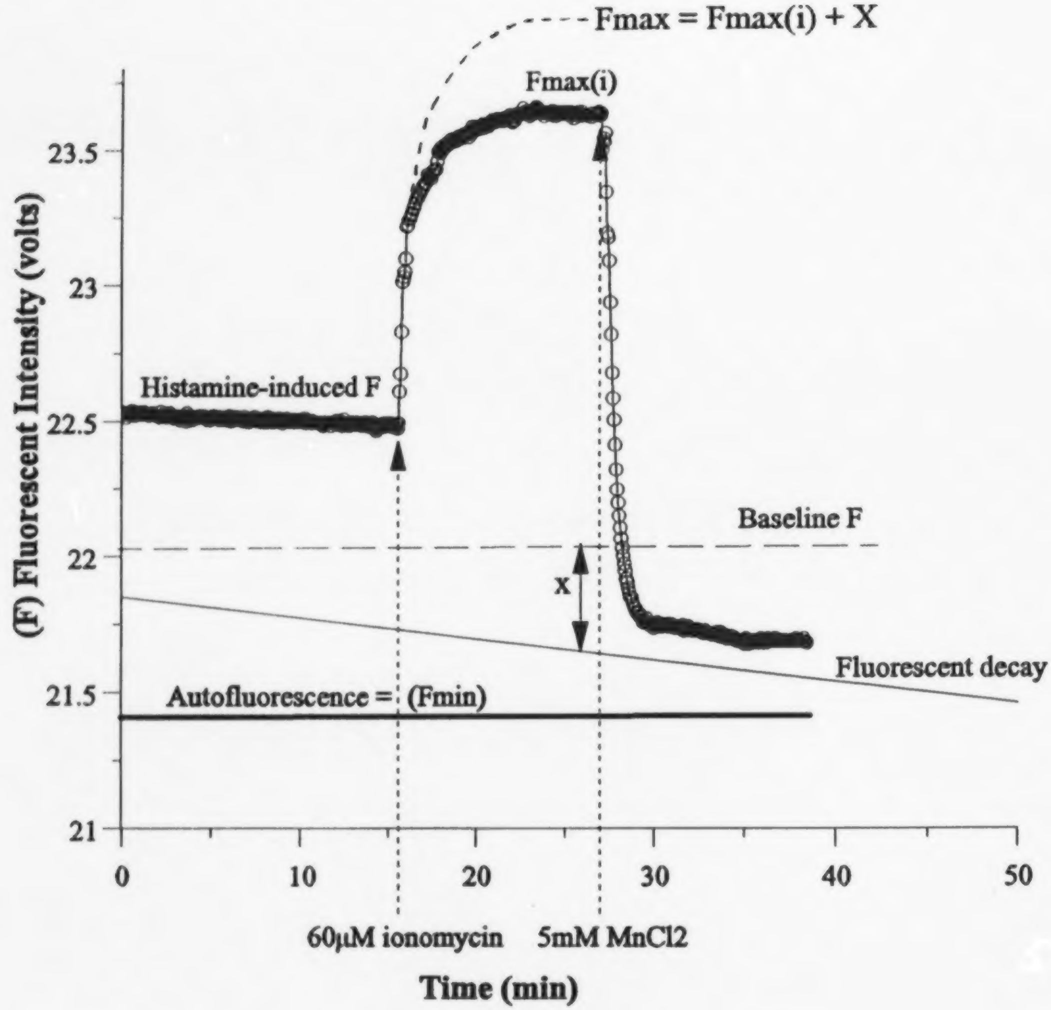

Figure #3.7 Calibration Curve for Fluo-3 loaded Trachealis Muscle

Effect of 60 μM ionomycin and 5 mM $MnCl_2$ on fluorescent intensity, ——— is the slope of fluorescent decay and - - - - is the baseline intensity; X is the change from baseline fluorescence to fluorescent decay at time Fmax(i).

Discussion

In this study I found that fluo-3 did not significantly alter histamine-induced isometric tension, and that fluo-3 is a useful indicator for measuring $[Ca^{++}]_i$ in bovine tracheal smooth muscle tissue. Furthermore, my experimental set-up permitted simultaneous measurements of $[Ca^{++}]_i$ and isometric tension. The present study showed that histamine caused a sustained increase in $[Ca^{++}]_i$ while maintaining an increased isometric tension.

The optimal concentration of fluo-3 AM in these tracheal smooth muscle tissue studies was 15 μM, and the optimal incubation period was 3 hours. With 3 hours of incubation with 5 to 10 μM concentrations of fluo-3 AM there were insignificant changes in fluorescent intensity with ionomycin (60 μM).

Baseline $[Ca^{++}]_i$ values were approximately 50 % higher than those reported by Gunst and Brandyopadhyay, (1989), but they used canine tissue and aequorin biolurninesense. The variation therefore could be attributed to species differences (bovine vs. canine) or to the technique for measuring $[Ca^{++}]_i$. It is also possible that high $[Ca^{++}]_i$ is observed in my studies from fluorescence other than the myoplasm F. The AM ester of fluo-3 enters the myoplasm by simple diffusion and the esterase in the cytosol hydrolyses the AM derivative to form the polycarboxylated anion (calcium sensitive salt) (Tsien, 1988). This process is straight forward in cultured cells but in the case of a tissue strip, damaged cells on the surface might cause a higher measured $[Ca^{++}]_i$ due to 1) free esterase being present on the cell surface resulting in excessive calcium/fluo-3 complex, and /or, 2) the damaged cells allow a abnormally high amount of calcium into the myoplasm. Our measurement was an average of $[Ca^{++}]_i$ within the tissue strip. The F could be theoretically increased with an increase in $[Ca^{++}]_i$, or with an increase in muscle cell volume in the light path. Against the change in $[Ca^{++}]_i$ being entirely due to the latter, I found that when exposed to ionomycin, $[Ca^{++}]_i$ increased more rapidly and to its maximum several minutes before maximum tension was achieved (data not shown).

Approximately 65 % of all experiments were accepted. To be accepted the tissue strip had to: 1) show a stable L_o before and after dye loading, 2) respond to histamine by generating an isometric contraction, and 3) have an $F_{max(i)}$ value higher than the F value during a contraction.

The sustained increase in $[Ca^{++}]_i$ during histamine-induced isometric contraction could result from a steady release of stored calcium from the sarcoplasmic reticulum, and/or from extracellular calcium entry via voltage operated calcium channels. During the initial histamine-induced tension increase the release of myoplasmic stored calcium is caused by increased IP_3 accumulation (Barnes *et al.*, 1986; Kotlikoff *et al.*, 1987). The sustained histamine contraction causes a depolarization of the membrane (Kirkpatrick, 1975) thus, activating voltage operated calcium channels (Rodger, 1988). The combination of the two calcium pools could therefore maintain the elevated intracellular calcium level. This two calcium pool hypothesis is substantiated by the tension studies of Kirkpatrick, (1975) and Fukui *et al.*, (1989) where extracellular calcium depletion experiments along with my isometric tension-low extracellular calcium study (chapter 2 fig 2.14) showed that removal of extracellular calcium concentration reduces the peak tension as well as removing the plateau. Even with the high concentration of fluo-3 AM used in this study, the calcium buffering capacity of fluo-3 had very little effect on the rate of rise of isometric contraction or the maximum contraction to histamine. These results are best appreciated in figures 3.2 and 3.3 where the smooth muscle rate of tension increase and maximum tensions between control and fluo-3 loaded tissue were not significantly different.

In summary, fluo-3 did not affect histamine-induced peak isometric tension in bovine tracheal smooth muscle, and it was useful in measuring changes in $[Ca^{++}]_i$. These results demonstrate that simultaneous measurement of $[Ca^{++}]_i$ and isometric tension in bovine tracheal smooth muscle can be achieved using fluo-3AM.

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Chapter IV

Effects of Salbutamol, Histamine and Acetylcholine on Calcium Mobilization, Cyclic AMP and Isometric Tension in Bovine Tracheal Smooth Muscle

Introduction

β_2-agonist inhalation therapy is one of the pharmacological modalities used to prevent or reverse bronchospasm. The mechanism of β_2-agonist relaxation in airway smooth muscle has not been clearly defined; however, the second messengers, adenosine 3', 5', cyclic monophosphate (cyclic AMP) and intracellular calcium have been closely associated with its contraction and relaxation (Tukawa *et al.*, 1987; Gunst, 1989; Rasmussen *et al.*, 1990; Bolton, 1979)

Cyclic AMP and Airway Smooth Muscle Relaxation

Pharmacological agents which cause an increase in cyclic AMP concentration have been shown to also relax airway smooth muscle *in vitro* (Bolton, 1979; Douglas and Brink 1987; Katsuki and Murad, 1976). The potential sites of action of this cyclic AMP-induced smooth muscle relaxation are: 1) phosphorylation of the myosin light chains, causing a decrease in sensitivity of calcium by myosin light chain kinase (Aksoy *et al.*, 1983; Stull *et al.*, 1988), 2) changes in intracellular calcium metabolism (calcium efflux, influx and reuptake by myoplasmic stores) causing a decrease in intracellular calcium concentration ($[Ca^{++}]_i$) (Itoh *et al.*, 1982; Meisheri *et al.*, 1982; Bulbring and den Hertog 1980; Scheid *et al.*, 1979; and Van Breeman, 1977) and 3) phospholipase C and inositol phospholipid kinases which inhibit the hydrolysis of inositol phospholipids which in turn modulate the histamine-induced contractile response (Hall *et al.*,1989). To address the second issue raised above, I studied the relationship between isometric contraction, intracellular cyclic AMP concentration, $[Ca^{++}]_i$ and extracellular calcium ($[Ca^{++}]_{ext}$) mobilization. Accordingly, I carried out simultaneous measurements of either 1) cyclic AMP, 2) $[Ca^{++}]_i$ or 3) $[Ca^{++}]_{ext}$ and isometric tension when bovine tracheal smooth muscle was contracted with histamine, or acetylcholine and relaxed with salbutamol.

Calcium and Airway Smooth Muscle Relaxation

Simultaneous measurement of $[Ca^{++}]_i$ and isometric tension in tracheal smooth muscle strips when stimulated with pharmacological agents has advanced our understanding on how calcium is involved in the development and reduction of tension in airway smooth muscle (Tukawa *et al.*, 1987; Gunst and Bandyopadhyay, 1989; Ozaki *et al.*, 1990). Gunst and Bandyopadhyay, (1989); Tukawa *et al.*, (1987) and Ozaki *et al.*, (1990) have reported a decrease in $[Ca^{++}]_i$ in precontracted tracheal smooth muscle when relaxed with a β-agonist, whereas, the reports on the effect of a β-agonist on resting airway smooth muscle are inconsistent; either causing an increase in $[Ca^{++}]_i$ (Tukawa *et al.*, 1987) or no effect (Gunst and Bandyopadhyay, 1989). Whereas, a reduction in $[Ca^{++}]_i$ is observed in smooth muscle relaxation, there have been no experiments to clearly delineate if the decrease in $[Ca^{++}]_i$ caused by β-agonist stimulation is associated with calcium efflux from the myoplasm in airway smooth muscle, or by other mechanisms.

To address the role of changes in calcium mobilization I used the fluorescent indicator fluo-3 in both the acetoxymethyl ester (AM) and the pentaammonium salt, to make: 1) simultaneous measurements of $[Ca^{++}]_i$ and isometric tension, and 2) simultaneous measurements of $[Ca^{++}]_{ext}$ and isometric tension. I determined previously in chapter 3 that fluo-3 AM can be used to simultaneously monitor $[Ca^{++}]_i$ and isometric tension in bovine tracheal smooth muscle and that, fluo-3 can detect calcium over a wide range, from 35 nM to a saturation concentration of approximately 10 µM, (Minta *et al.*, 1989; Kao *et al.*, 1989). Monitoring small changes in calcium surrouding the tissue ($[Ca^{++}]_{ext}$) during histamine-induced contraction and/or salbutamol-induced relaxation requires fluo-3 salt to present in the bathing solution, and that the modified Krebs-Henseleit solution contains a calcium concentration of approximately 700 nM (Stockbridge, 1987). These two factors will facilitate the detection of any significant changes of $[Ca^{++}]_{ext}$ during contraction and/or relaxation of the tissue strip.

In this chapter I am **reporting** on the effects of salbutamol, histamine or acetylcholine on calcium mobilization (intra and extracellularly) or on cyclic AMP concentration, simultaneously with isometric tension.

Materials and Methods

Fluo-3 acetoxymethyl ester (AM) and fluo-3 pentaammonium salt were purchased from Molecular Probes Inc. (Eugene, OR, USA); Pluronic F-127 was supplied by Molecular Probes. Histamine phosphate, $MnCl_2$, trichloroacetic acid and a protein analysis kit (P5656) were purchased from Sigma (St. Louis, MO. USA). Acetylcholine chloride was purchased from Fluka Chemika (AG., Switzerland). Salbutamol sulphate was donated from Glaxo Canada Inc. (Toronto, OT). Ionomycin was purchased from Terochem Laboratories (Edmonton, AB. CANADA). Cryogenic tubes were purchased from Fisher Scientific (Nepean, ON. CANADA). A cyclic AMP assay kit (TRK.432) was purchased from (Amersham, International, UK). Two Krebs-Henseleit buffers were used in these experiments, 1) normal Krebs-Henseleit (KH) containing (in mM) 113 NaCl, 4.8 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, and 5.5 glucose; 2) modified Krebs-Henseleit (MKH) 113 NaCl, 4.8 KCl, 0.38 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, 5.5 glucose, and 0.41 EGTA. The MKH buffer gives a total free calcium concentration of $\simeq$ 700 nM (Stockbridge, 1987).

Smooth Muscle Isolation

This method was similar to that described in chapter II with the following additions: the tracheal strips used for $[Ca^{++}]_{ext}$ experiments were placed in MKH solution at 4 °C, and were approximately 2 mm wide x 2 mm thick x 10 mm long (wet weight was 20 to 40 mg).

Simultaneous [Ca++]$_i$ and Isometric Tension Measurement

In addition to the contractile agent (histamine) used in chapter III, the effects of salbutamol (1 μM) (Hall *et al.*, 1989) were tested on muscle strips at Lo and on muscle strips contracted by histamine either (3.5 μM) or acetylcholine (0.5 μM).

Cyclic AMP and Isometric Tension Measurement

Muscle strips were placed in cryogenic tubes (2 mls), bathed in KH solution which was oxygenated with 95 % O_2 and 5 % CO_2 and the bicarbonate buffer in the KH solution maintained the pH at 7.4. The temperature was maintained at 37 °C. One end of the muscle strip was attached firmly to the base of the tube using a metal clip, the other, to a force transducer (FT-03; Grass Instrument, Quincy, MA, USA). This set-up is similar to figure 2.2 in chapter II except that cryogenic tubes were mounted inside the tissue baths so that the tissue could be rapidly frozen. As electric field stimulation was not used to establish the active resting tension (Lo), I used a method similar to that described by Marthan *et al.*, (1987). The muscle length was increased very slightly every 5 to 10 minutes for a total of approximately 90 minutes until resting tension stabilized.

The muscle strips were then subjected to one of the procedures described below, following which they were immediately frozen using 1.8 mls of liquid nitrogen added directly to the cryogenic tube. The tissue was (**see figure 4.1**): 1) pretreated for 10 min. with salbutamol (1 μM) (**A'**) or isobutyl-methyl xanthine (IBMX) (a non-specific phosphodiesterase) (0.1 mM) (**A**) (Hall *et al.*, 1989), 2) stimulated with histamine 3.5 μM and frozen at 2 (**B**) or 7 (**C**) minutes, or 3) stimulated with histamine 3.5 μM for 10 minutes and relaxed with salbutamol (1 μM) and frozen at 10 minutes (**D**). It took approximately 10 seconds to empty the KH solution from the cryogenic tube and replace it with liquid nitrogen. Throughout these procedures mentioned above(**A', A, B, C, D**) muscle tension was monitored.

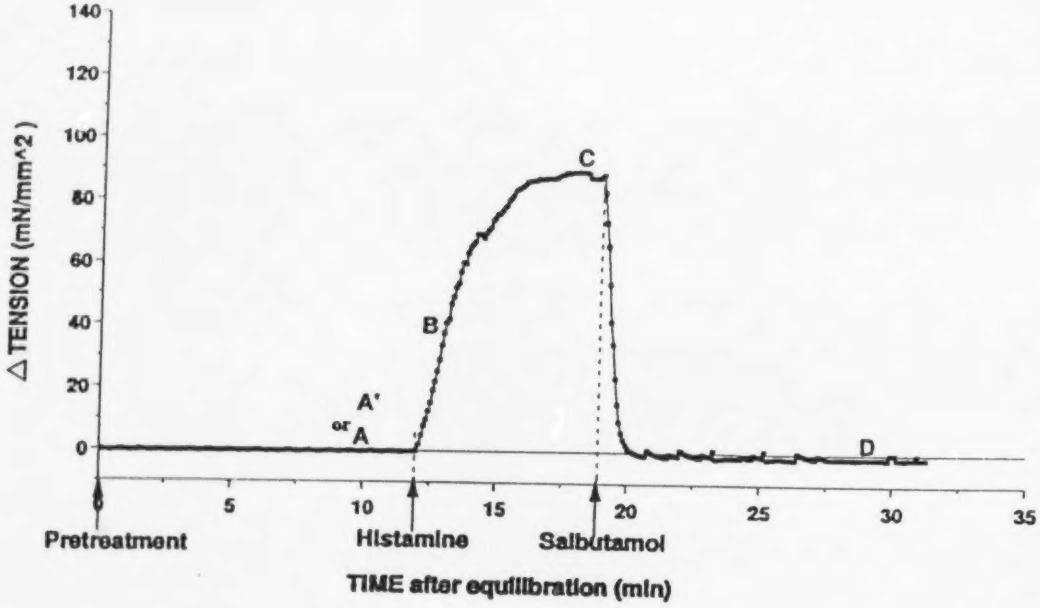

Figure # 4.1 Time Points for Cyclic AMP Measurement

For the measurement of cyclic AMP, the tissues were processed at 4 °C or lower, to inhibit changes in cyclic AMP concentration caused by cytosolic phosphodiesterases. Frozen muscle strips were weighed and minced using a scalpel blade. The minced tissue was placed in 400 μL of a 6% solution of trichloroacetic acid (TCA) and homogenized at 40,000 rpm for 1 minute. (conical homogenizer VIRTIS). The homogenate was centrifuged at 1500 g for 10 minutes at 4 °C. The supernatant was collected, and the TCA extracted using 5 successive rinses with ether saturated with water (Brooker *et al.*, 1979). The precipitate was saved and the protein content measured using a method similar to that described by Lowry *et al.*, (1951). The cyclic AMP concentration for each muscle strip was then determined using the ^3H assay kit and a scintillation counter (Beckman LS 7000), with appropriate quenching and background correction.

Simultaneous $[Ca^{++}]_{ext}$ and Isometric Tension Measurement

Muscle strips were placed in the CAF-100 identical to the method described in chapter III. The tissue was bathed in MKH solution which was oxygenated with 95 % O_2 and 5 % CO_2 at a pH of 7.4. The temperature was maintained at 37 °C. The Lo was achieved identical to that described for cyclic AMP and isometric tension experiments described above. The MKH solution contained 700 nM free calcium and was the minimum amount of calcium that would allow for a measurable histamine-induced contraction after 2 hours bathing time. The simultaneous and continuous measurements of $[Ca^{++}]_{ext}$ and isometric tension were made after fluo-3 pentaammonium salt (15 μM) (a cell impermeant) was added to the MKH solution. The data recorded consisted of continuous measurements of fluorescent intensity and isometric tension over a 15 minute period. To show the timing seqeuncing of changes that occured during agonist stimulation both measurements were plotted in arbitrary units.

The experimental protocol was as follows (see figure 4.5): baseline values (fluorescent intensity and isometric tension) were recorded for 3 minutes, thereupon double distilled water ($H_2O_{(dd)}$) (15 µl) was added determining the effects of dilution artifact on fluorescent intensity and values were recorded for a further 2 minutes. At 5 minutes, 15 µl histamine (10 µM) (ED80) was added and measurements were carried out for 5 minutes. This higher concentration of histamine was required to produce a brisk contraction in the lower calcium buffer. At 10 minutes, 15 µl salbutamol (1 µM) was added and the effects were recorded for 3 minutes thereafter. To indicate if fluo-3 was saturated $CaCl_2$ solution (15 µl) for a final concentration of $\simeq$ 5 µM free calcium was administered 3 minutes after the addition of salbutamol for each tissue strip.

Statistical Analysis

Baseline values for $[Ca^{++}]_i$ when the muscle was at L_o are presented as the mean ± SEM. Cyclic AMP values are presented as picomoles per milligram of protein. Comparisons between cyclic AMP concentration of control (muscle strips at Lo) and stimulated muscle strips were using the paired *t* test. Significance was defined as $P \leq 0.05$.

Results

Effects of Salbutamol on $[Ca^{++}]_i$ and Isometric Tension During Contraction (caused by histamine or ACh) or at L_o

Table 4.1 shows the results of 7 trachealis muscle strips exposed to salbutamol (1 μM). Regardless of whether the tissue was precontracted by histamine (3.5 μM) (n=3) or acetylcholine (0.5 μM) (n=2) or whether it was maintained at L_o (n=2), salbutamol caused $[Ca^{++}]_i$ and tension (in precontracted tissue) to decline. Figure 4.2 is a typical example of a histamine (3.5 μM)-induced response which caused a rapid increase in $[Ca^{++}]_i$ to a peak value followed by a moderate decline, the rate of which was similar to that seen during the baseline period. Furthermore, salbutamol (1 μM) caused a rapid decline in the elevated $[Ca^{++}]_i$, returning it to a value near baseline. Histamine caused a slow increase in isometric tension which reached a plateau. This tension was abolished after the addition of salbutamol (1 μM).

ACh (0.5 μM) caused a rapid rise in $[Ca^{++}]_i$ which was either transient or sustained, and the addition of salbutamol (1 μM) caused $[Ca^{++}]_i$ to decrease rapidly. Furthermore, the ACh caused isometric tension to increase rapidly to a plateau which was abolished with the addition of salbutamol (1 μM) (data not shown).

Figure 4.3 shows a decrease in $[Ca^{++}]_i$ from baseline values when salbutamol (1 μM) was administered to the muscle at L_o.

	Baseline		**Max. $[Ca^{++}]_i$ and IT**		**Post Salbutamol**	
Tissue	$[Ca^{++}]_i$ (nM)	IT (mN/mm^2)	$[Ca^{++}]_i$ (nM)	IT $\Delta(mN/mm^2)$	$[Ca^{++}]_i$ (nM)	IT $\Delta(mN/mm^2)$*
1	393.3	0.1	1171.2	117.9	318.8	-5.3
2	370.2	0.6	785.2	164.4	225.9	-51.9
3	360.2	0.0	853.6	103.2	45.3	-4.3
4	379.7	0.0	584.0	74.9	330.0	-2.8
5	437.6	0.1	683.4	160.3	332.5	-4.2
6	334.4	0.0			114.0	-6.9
7	452.6	-0.2			195.5	-16.8
	389.7±15.9	0.1±0.1	**(mean ± SEM)**			

*negative values symbolize below baseline values.

Tissues 1 to 3 were contracted with 3.5 μM histamine and relaxed with 1.0 μM salbutamol (See figure #4.2).

Tissues 4 to 5 were contracted with 0.5 μM acetylcholine and relaxed with 1.0 μM salbutamol.

Tissues 6 to 7 were stimulated with 1.0 μM salbutamol (See figure #4.3).

Table #4.1 Effects of Salbutamol on $[Ca^{++}]_i$ and Isometric Tension (IT)

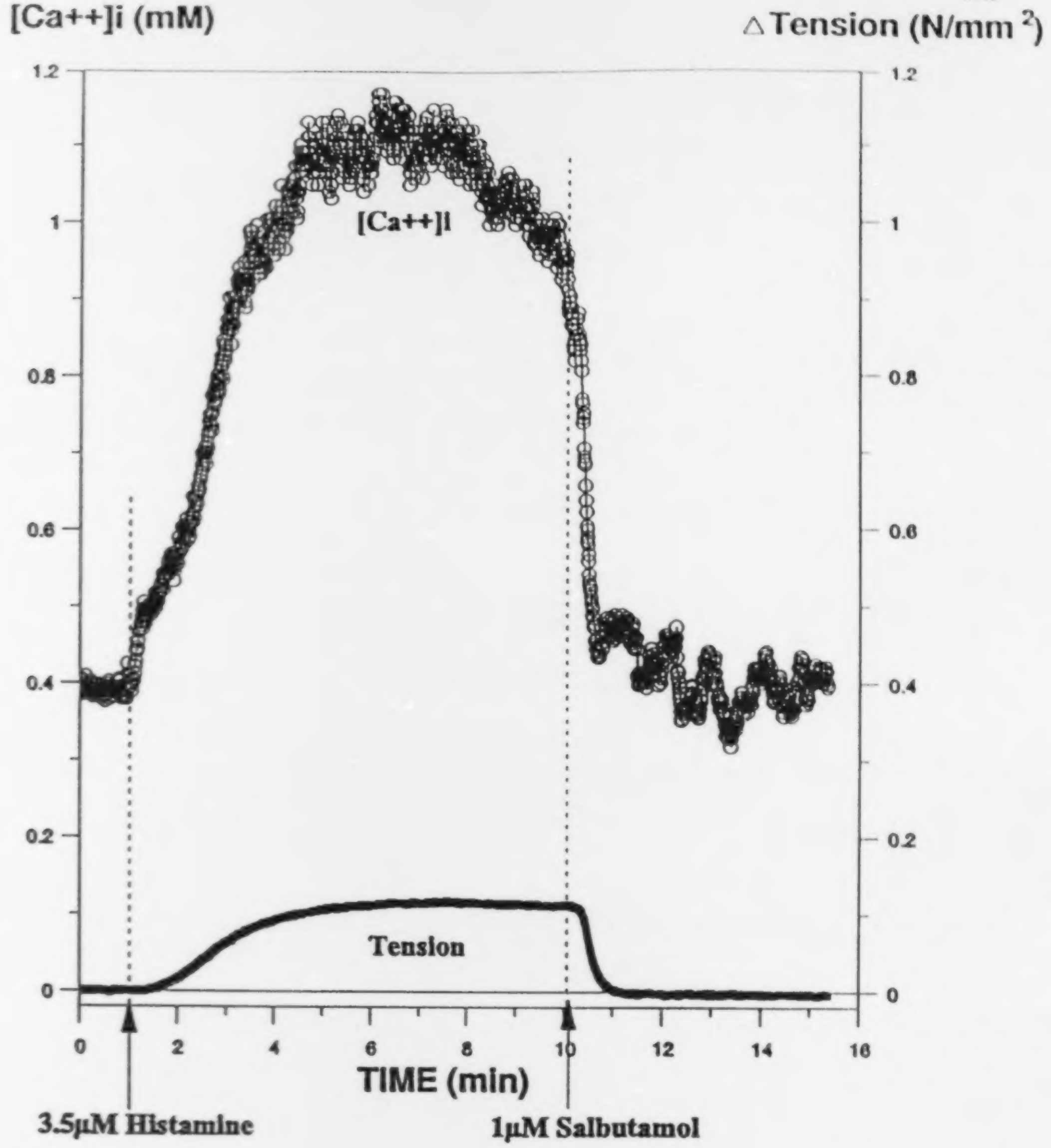

Figure #4.2 Effect of Histamine and Salbutamol on Simultaneous $[Ca^{++}]_i$ and Isometric Tension in a Bovine Trachealis Muscle.

At the 1 minute mark histamine (3.5 µM) was added and at the 10 minute mark salbutamol (1 µM) was added. $[Ca^{++}]_i$ is the upper tracing, isometric tension is the lower tracing. Data acquisition was at 2 samples/second.

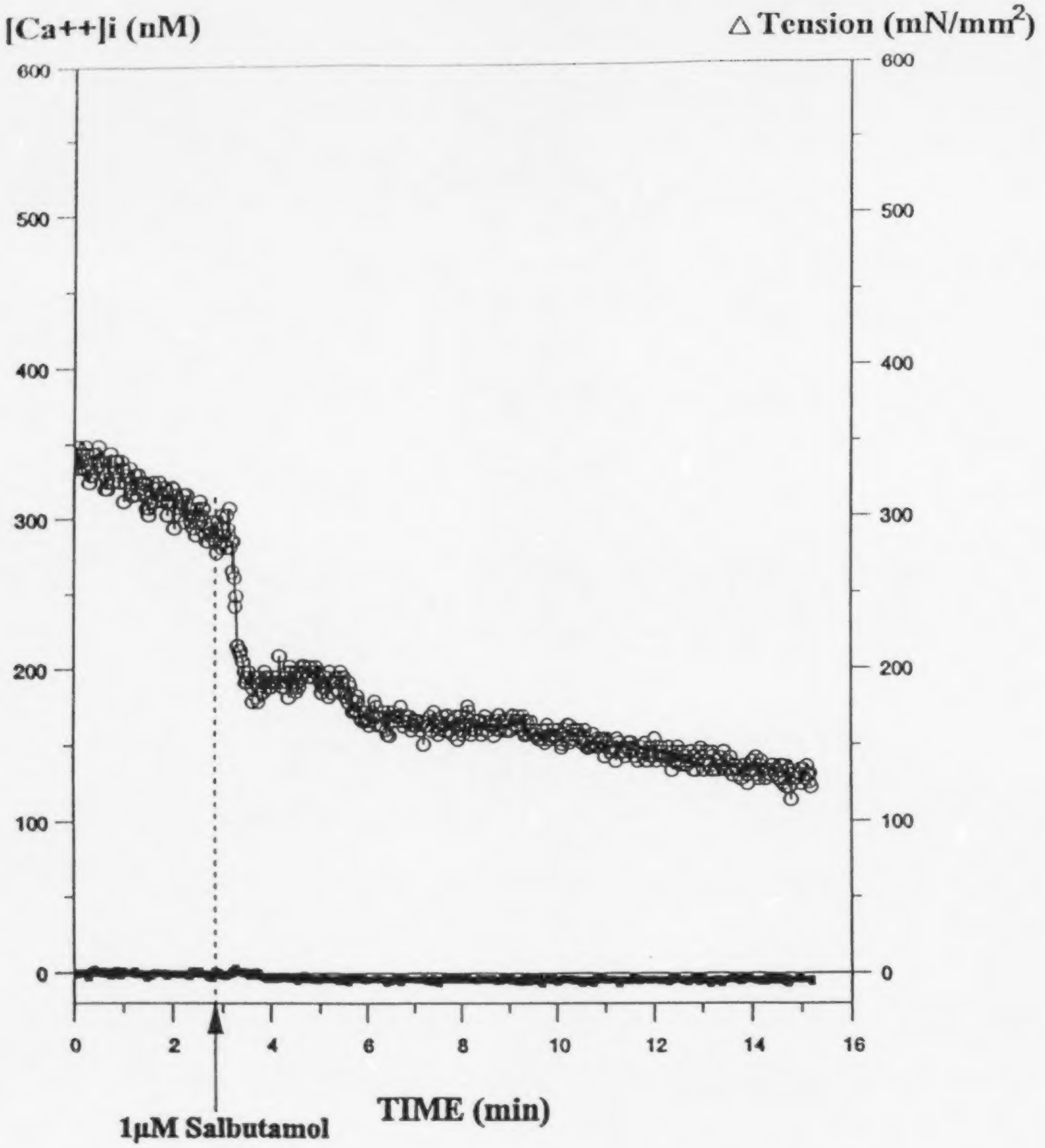

Figure #4.3 Effect of Salbutamol on Simultaneous $[Ca^{++}]_i$ and Isometric Tension in a Bovine Trachealis Muscle.

At the 3 minute mark salbutamol (1 μM) was added. $[Ca^{++}]_i$ is the upper tracing, isometric tension is the lower tracing. Data acquisition was at 2 samples/second

Effects of IBMX, Histamine, Salbutamol on Cyclic AMP Concentration

Figure 4.4 shows the effects of IBMX (0.1 mM), salbutamol (1 μM), histamine (3.5 μM) and histamine and salbutamol (1 μM) on cyclic AMP concentration in bovine tracheal smooth muscle at various tensions (see figure 4.1 for time points) (n=39). The average cyclic AMP concentration in 11 trachealis muscle strips at L_o (control) was 5.32 pmol/mg protein. Pretreatment for 10 minutes with IMBX (0.1 mM) caused an increase in cyclic AMP concentration which was significantly higher than control ($n=5$, $P \leq 0.05$). Pretreatment for 10 minutes with salbutamol (1 μM) caused an increase in cyclic AMP concentration which was significantly higher than control ($n=6$, $P \leq 0.05$). After 2 minutes exposure to histamine (3.5 μM), there was an increase in cyclic AMP concentration which was significantly higher than control ($n=8$, $P \leq 0.05$). However, after a 10 minute pretreatment with histamine (3.5 μM) there was no significant difference from control (n=5). Ten minutes after salbutamol (1 μM) was added, to tissues already contracted by histamine, there was an increase in cyclic AMP concentration which was significantly higher than control ($n=4$, $P \leq 0.05$).

There was no significant difference in cyclic AMP concentration whether the control tissues were frozen with liquid nitrogen or frozen in a -80 °C fridge (n=4). Therefore, the mode of freezing did not influence our data.

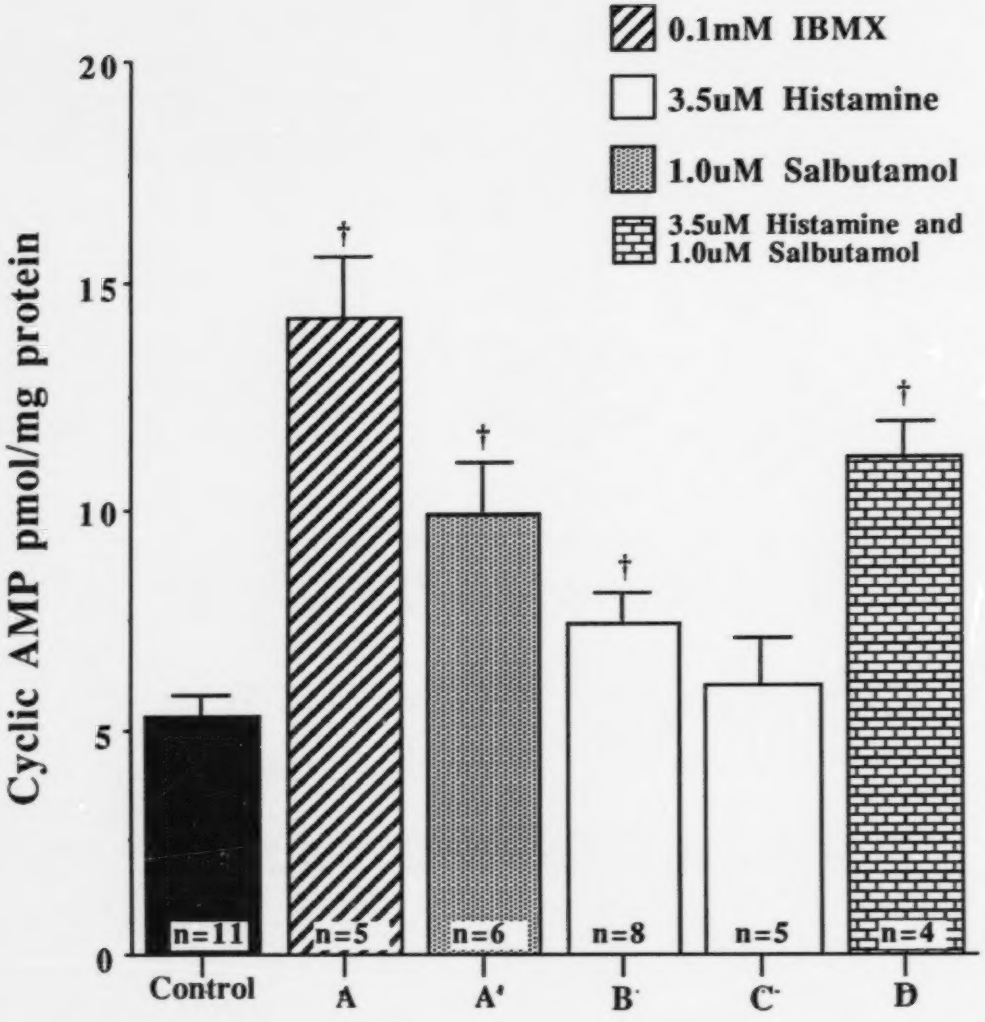

Figure #4.4 Effects of IBMX, Salbutamol, Histamine and Histamine with salbutamol, on Cyclic AMP Concentration in Bovine Trachealis Muscle.
Time points for cyclic AMP: A and A' were pretreated for 10 minutes, B was at 2 min. during a contraction caused by histamine, C was at 10 min. during a contraction caused by histamine, D was at 10 min. after a contraction caused by histamine and relaxed with salbutamol (see figure 4.1). † indicates significance from control $P \leq 0.05$.

Effects of Histamine and Salbutamol on $[Ca^{++}]_{ext}$ and Isometric Tension.

The effects of $H_2O_{(dd)}$ (15 µl), histamine (10 µM), and salbutamol (1 µM) on fluorescent intensity of the bathing medium (indicative of $[Ca^{++}]_{ext}$) and isometric tension of 1 of 4 tissue strips is shown in (figure 4.5). The changes in $[Ca^{++}]_{ext}$ caused by histamine and salbutamol are typical of those seen in the other three tissues studied. Addition of 15 µl H_2O(dd) (the same volume dose used for histamine and salbutamol) had no affect on isometric tension but caused a slight decrease in $[Ca^{++}]_{ext}$ (**A**) because of the dilution effect. Histamine (10 µM) initially caused a slight increase in $[Ca^{++}]_{ext}$ (**B**) followed by a decrease in the slope (**C**). The isometric tension increased steadily approximately 30 seconds after the administration of 10 µM histamine (**D**). Salbutamol (1 mm) caused an immediate increase in $[Ca^{++}]_{ext}$ (**E**) which was associated with a decrease in isometric tension. Addition of 15 µl $CaCl_2$ to achieve ≃ 5 µM in the bathing medium caused a rise in fluorescent intensity similar to the increase observed during the stimulation with 1 µM salbutamol (**F**).

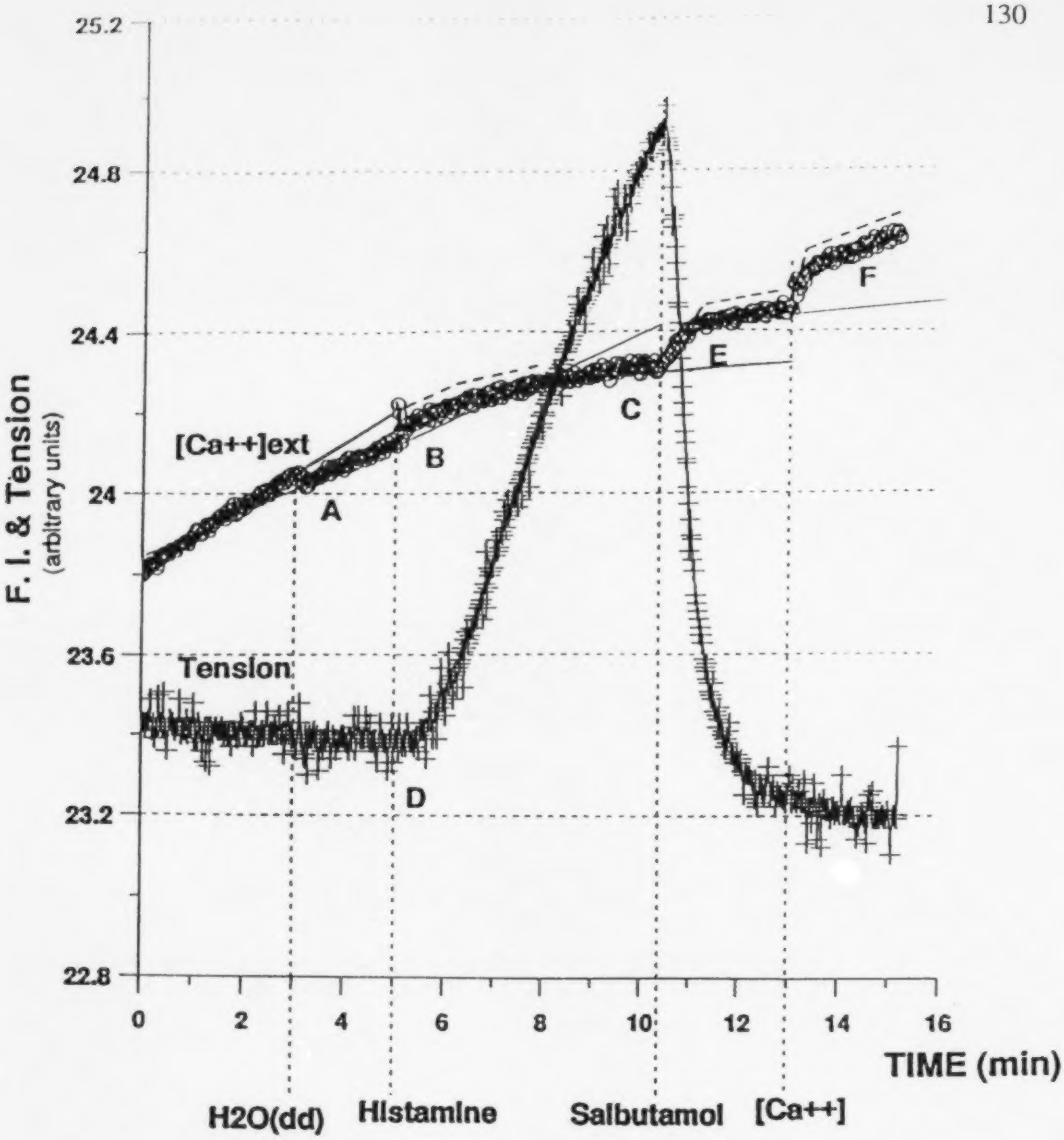

Figure #4.5. Effect of H_2O, Histamine and Salbutamol on Fluorescent Intensity and Isometric Tension in a Bovine Trachealis Muscle.

At the 3 minute mark 15 μl H_2O (dd) was added, at 5 minutes histamine (10 μM), at 10.4 minutes salbutamol (1 μM) and at 13 minutes $CaCl_2$ solution (4.3 μM) was added. $[Ca^{++}]_{ext}$ fluorescent intensity (F.I.) is depicted by open circles, isometric tension is depicted by crosses. (-----) represents the change in F.I. caused by the dilution effect. Data acquisition was at 2 samples/second.

Discussion

In this study I have demonstrated in a preliminary fashion, for the first time, that salbutamol caused a decrease in baseline $[Ca^{++}]_i$ in bovine tracheal smooth muscle at L_o. Also, salbutamol is associated with an increase in $[Ca^{++}]_{ext}$ when added during a histamine-induced contraction. Furthermore, salbutamol caused a significant increase in cyclic AMP concentration in tissues which were at L_o or precontracted with histamine.

Takuwa *et al*., (1987) reported that isoproterenol caused a transient increase in $[Ca^{++}]_i$ in aequorin loaded resting bovine trachealis muscle strips. Felbel *et al*., (1988) also found an isoproterenol-induced increase in $[Ca^{++}]_i$ in bovine tracheal smooth muscle cells using fura-II as the $[Ca^{++}]_i$ indicator. However, Gunst and Brandyopadhyay, (1989) and Ozaki *et al*., (1990), found that isoproterenol did not increase $[Ca^{++}]_i$ in resting aequorin and fura-II loaded canine trachealis muscle strips. This inconsistency in the literature combined with the salbutamol-induced decrease in $[Ca^{++}]_i$ when the tissue is at L_o, which I recorded, brings some uncertainty to the hypothesis proposed by Rasmussen *et al*., (1990), which is that an increase in $[Ca^{++}]_i$ can play a dual role: it could mediate both contraction and relaxation in airway smooth muscle. Differences between my observations and those reported previously may be in part due to species variation (bovine vs. canine), temperature and/or $[Ca^{++}]_i$ monitoring technique (fluorescence vs. bioluminescence). However, the decrease in $[Ca^{++}]_i$ caused by the addition of salbutamol to histamine or acetylcholine contracted tracheal smooth muscle, is similar to the results of Takuwa *et al.*, (1987); Felbel *et al.*, (1988); Gunst and Brandyopadhyay, (1989); Ozaki *et al.*, (1990).

The increase in cyclic AMP concentration caused by salbutamol in my study was similar to that reported by Hall *et al.*, (1989) and support the hypothesis that a rise in cyclic AMP is associated with airway smooth muscle relaxation (Gold *et al.*, 1980). However, the significant increase in cyclic AMP concentration in the first two minutes following histamine stimulation is similar to the results of Katsuki and Murad, (1977); and Andersson *et al.*, (1975). Therefore, these results suggest a complex interaction between cyclic AMP and $[Ca^{++}]_i$ (Rasmussen *et al.*, 1990). This transient rise in cyclic AMP concentration, caused by histamine, may be the result of local histamine-induced synthesis of prostaglandin, or a histamine-induced increase in cyclic GMP which could then cause a secondary increase in cyclic AMP (Stoner *et al.*, 1974). Alternatively, histamine could cause activation of the H2 receptor which could then initiate a transient increase in cyclic AMP (Douglas, 1985).

The simultaneous measurement of extracellular calcium and isometric tension revealed two important observations: 1) histamine, during the initial rise in tension, resulted in a very slight transient increase in $[Ca^{++}]_{ext}$, but as the tension approached a plateau, the slope of the rate of increase in $[Ca^{++}]_{ext}$ declined, 2) salbutamol caused an instantaneous increase in $[Ca^{++}]_{ext}$ and a decrease in isometric tension. These results suggest that the action of histamine: 1) during the initial tension increase did not require a supply of calcium from the bathing medium and supports the findings by Kirkpatrick, (1975) Takuwa *et al*., (1987); Kotlikoff *et al.*, (1987), and 2) when approaching the sustained component of isometric contraction requires a supply of calcium from the bathing medium. This influx of calcium may be secondary to a depolarization of the membrane (Kirkpatrick, 1975) causing activation of voltage dependant calcium channels (Rodger, 1987). The $[Ca^{++}]_{ext}$ increase and isometric tension decrease caused by salbutamol establishes a possible mechanism similar to that described by (Scheid *et al*., 1979) that β-agonist-induced relaxation is caused by an increase in Na^+-K^+ pump activity, which increases the transmembrane Na^+ gradient and enhances the calcium extrusion via the Na^+-Ca^{++} exchanger, or that calcium is effluxed by a direct activation of the plasmalemma calcium pump working in parallel with the Na^+-Ca^{++} exchanger (DiPolo and Beauge, 1990).

One other noteworthy point is that the fluorescent intensity increased at a constant rate throughout the extracellular calcium/isometric tension measurement. This could be the effect of adding a calcium buffer (fluo-3) to the bathing medium causing a steady efflux of calcium from the cells of the tissue and /or from temperature variation during installation of the dye.

In summary, my results indicate that salbutamol administered before or during a histamine-induced contraction caused an increase in cyclic AMP, a decrease in $[Ca^{++}]_i$ and an increase in $[Ca^{++}]_{ext}$. These findings suggest that the cyclic AMP action may be linked to calcium efflux from the myoplasm.

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Chapter V

Conclusion

Conclusion

Airway smooth muscle is found throughout the tracheobronchial tree and is one of the major factors that dictate the flow of air entering or leaving the respiratory zone during inspiration and expiration. Regulation of the tone in airway smooth muscle is a dynamic state between various excitatory and inhibitory mechanisms operated by second messenger systems. This thesis focused on how calcium and cyclic AMP, both second messengers, control the isometric contraction and relaxation of airway smooth muscle. In this final chapter a brief background of the topics of each study will be reviewed along with a succinct description of the results of each chapter followed by a general conclusion.

Chapter II

Inflammatory mediators leukotriene D_4 (LTD_4), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), platelet-activating factor (PAF) and histamine have been linked to the pathogenesis of asthma (Abrahams and Wanner, 1988) and have been shown to cause airway smooth muscle contraction *in vivo* (Kern *et al.*, 1986; Denjean *et al.*, 1983; Malo *et al.*, 1982). In this chapter, I have shown that LTD_4, $PGF_{2\alpha}$, PAF and histamine all caused tracheal smooth muscle isometric contraction, but the peak tensions obtained for LTD_4, $PGF_{2\alpha}$ and PAF were variable and the tension-time curves difficult to interpret for reasons discussed below.

The effect of **LTD_4** on peak tension in bovine trachealis muscle was variable and the isometric tension-time curve showed a transient increase in tension. Also, the LTD_4-induced contraction showed no change in tension when acetylcholine (ACh) was administered after maximum tension via LTD_4, but once the tissue was rinsed with Krebs-Henseleit (KH) solution, the same dose of ACh caused a brisk and maintained tension. This suggests that LTD_4 could cause one or more of the following: 1) the contractile elements decrease their calcium sensitivity (Karaki, 1989), 2) release of vasoactive intestinal polypeptide, or release of prostaglandin E_2, both of these compounds are found *in vivo* and have been shown to cause airway smooth muscle relaxation (Said, 1988; Madison *et al.*, 1989), and 3) some other unknown mechanism(s). These characteristics of the response to LTD_4 have not been reported in the literature and should be examined further.

The effect of **$PGF_{2\alpha}$** on bovine trachealis muscle was a maintained tension unlike that of either LTD_4 or PAF, and it was not tachyphylactic which was opposite to the results obtained using guinea pig trachealis preparations (Douglas and Brink, 1987). Furthermore, the maximum tensions generated with $PGF_{2\alpha}$ did not follow a well defined dose response.

The effect of **PAF** on bovine trachealis muscle was an isometric contraction without the addition of platelets to the bathing medium, and the response was tachyphylactic. PAF has been demonstrated to cause its contractile response through the activation of platelets which must be present for any contraction to occur (Popovich *et al.*, 1988; Schellenberg *et al.*, 1983; Morley *et al.*, 1989). Therefore, it is possible that some platelets were indeed present in the preparation, either adhering to the tissue strip or within the tissue strip. It is also possible that PAF-specific receptors exist on the trachealis muscle which mediate a contractile response in the absence of platelets.

The effect of **Histamine** on bovine trachealis muscle was a sustained tension that was dependant on extracellular calcium. Also the peak tensions recorded when using the same concentration of histamine showed no signs of tachyphylaxis. It is hoped that my work will stimulate further study.

Chapter III

Simultaneous measurement of $[Ca^{++}]_i$ and isometric tension in tracheal smooth muscle has advanced our understanding of how calcium is involved in the development and reduction of tension in airway smooth muscle, when exposed to certain pharmacological agents (Tukawa *et al.*, 1987; Gunst and Bandyopadhyay, 1989; Ozaki *et al.*, 1990).

Although the role of calcium in airway smooth muscle function is not completely understood, the generally excepted theory of how it regulates contraction is as follows: First, there is a rise in free intracellular calcium (activator calcium) which can come from either myoplasmic stores (sarcoplasmic reticulum) or from the extracellular space via calcium specific channels. Second, the free activator calcium binds to calmodulin, a specific myoplasmic binding protein. Third, the calcium/calmodulin complex interacts with myosin light chain kinase causing the kinase to phosphorylate and increase actinomyosin ATPase which results in cross-bridge cycling (Cheung, 1980; De Lanerolle, 1989). The rise in

$[Ca^{++}]_i$ caused by certain agonists, has been shown to be transient while a sustained tension is recorded (Gunst and Bandyopadhyay, 1989) and its pathway is not well understood.

Monitoring $[Ca^{++}]_i$ in airway smooth muscle tissue has been done using two techniques, aequorin bioluminescence and fura-II fluorescence. Due to problems associated with these two methods (reproducibility and quantitative calcium measurements), I have attempted measuring $[Ca^{++}]_i$ using a recently developed fluorescent indicator, fluo-3. In this chapter, I have found that fluo-3 could monitor changes in $[Ca^{++}]_i$ and that it did not affect either the rate of tension development, or peak tension caused by exposure to histamine. Analysis of the continuous $[Ca^{++}]_i$ and isometric tension data showed that histamine stimulation caused an initial increase in $[Ca^{++}]_i$ followed by a slow increase in isometric tension, and both $[Ca^{++}]_i$ and isometric tension were maintained at increased concentration and tension, respectively. It is hypothesized that histamine-induced elevated calcium levels occurred through a combination of two calcium sources. During the initial rise in tension, $[Ca^{++}]_i$ is supplied primarily from myoplasmic stores (Kotlikoff *et al*., 1987) and during the sustained contraction, $[Ca^{++}]_i$ is maintained by extracellular calcium entering from calcium voltage operated channels. The work completed in chapters II, III and IV, as well as the work by Kirkpatrick, (1975) and Rodger, (1987), support this hypothesis.

The pilot work done to obtain reproducible $[Ca^{++}]_i$ data was very extensive, and the established procedure using fluo-3 in this thesis could be considered for future studies involving simultaneous $[Ca^{++}]_i$ and isometric tension in airway smooth muscle or other tissue types.

Chapter IV

The interrelationship between $[Ca^{++}]_i$, cyclic AMP concentration, and isometric tension in airway smooth muscle when contracted or relaxed has been described as multidimensional (Rasmussen *et al.*, 1990). The results obtained while simultaneously measuring $[Ca^{++}]_i$ and isometric tension in airway smooth muscle, using various pharmacological agents and $[Ca^{++}]_i$ recording techniques (bioluminescence, fluorescence), have been inconsistent. However, one consistent finding is that β-agonists decreased the $[Ca^{++}]_i$ when the muscle was in the contracted state (Gunst and Bandyopadhyay, 1989; Tukawa *et al.*, 1987; Ozaki *et al.*, 1990). How this decrease in $[Ca^{++}]_i$ occurred in airway smooth muscle tissue has not been clearly established.

The importance of measuring extracellular calcium ($[Ca^{++}]_{ext}$) during a relaxation elicited by salbutamol is that it might provide insight into whether the calcium is sequestered by myoplasmic stores or effluxed to the extracellular or interstitial space. A possible pathway for the calcium is by direct or indirect efflux from the cells. ^{45}Calcium efflux experiments on cultured stomach smooth muscle cells showed that calcium efflux increased when the cells were treated with isoproterenol (Scheid and Fay, 1984). By adding the fluo-3 pentaammonium salt (the cell impermeant form) to the KH solution, I was able to simultaneously measure changes in $[Ca^{++}]_{ext}$ and isometric tension in bovine tracheal smooth muscle.

In this chapter, I have shown the effect of salbutamol on: $[Ca^{++}]_i$, $[Ca^{++}]_{ext}$, cyclic AMP concentration and isometric tension. I have found that salbutamol abolished both histamine or ACh-induced increases in both $[Ca^{++}]_i$ and isometric tension. Also, salbutamol caused a decrease in $[Ca^{++}]_i$ with no effect on tension when the tissue strip was at active resting tension (L_o). This effect of β-agonists on $[Ca^{++}]_i$ when the tracheal smooth muscle was at L_o has not been reported previously and is contradictory to the studies performed by Takuwa *et al.*, (1987) who have found that exposure to isoproterenol caused an increased $[Ca^{++}]_i$ in bovine tracheal smooth muscle. The results obtained Takuwa *et al.*, (1987) were not duplicated by Gunst and Bandyopadhyay, (1989) using the same technique and the same β-agonists. Furthermore, Ozaki *et al.*, (1990) found no change in $[Ca^{++}]_i$ in resting canine trachealis muscle when stimulated with isoproterenol.

The **cyclic AMP** concentration increased when salbutamol was administered to either airway smooth muscle at L_o or to histamine contracted smooth muscle. These results support the hypothesis that β-agonist cause relaxation by elevation of cyclic AMP only (Metzler and Kemp, 1991). However, it is unlikely that an increased cyclic AMP concentration is the sole cause for β-agonist relaxation, because of the results recorded by Andersson and Nilsson, (1978) and Katsuki and Murad, (1976), and the transient increase in cyclic AMP concentration caused by histamine as reported in this chapter. A possible mechanism by which histamine caused an increase in cyclic AMP concentration is either via the release of prostaglandins or by activating the H2 receptor (Stoner *et al.*, 1974; Douglas, 1985).

The $[Ca^{++}]_{ext}$ was slightly increased during the initial tension during a histamine-induced contraction, whereas the sustained tension showed a decrease in $[Ca^{++}]_{ext}$. Salbutamol caused an increase in $[Ca^{++}]_{ext}$ and a decrease in isometric tension which supports the theory that calcium efflux from the cytoplasm is a factor causing relaxation in smooth muscle (Van Breeman, 1977; Bulbring and der Hertog, 1980; Scheid and Fay, 1984).

In the process of completing this study I have added a new methodology which allows the simultaneous measurement of $[Ca^{++}]_{ext}$ and isometric tension. In so doing, I have added to the body of knowledge on the mechanism of action of β-agonist-induced relaxation of airway smooth muscle.

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